
THE UNIVERSITY *of* EDINBURGH

# How does pulmonary exposure to particulate matter predispose the heart to increased injury after myocardial infarction?

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# Declaration

I hereby declare that all work described in this thesis was performed by myself, except where otherwise acknowledged. This thesis has been composed entirely by myself and has not been previously submitted for any other degree or qualification.

Sarah Robertson

# Abstract

One of the most prevalent pollutants in urban cities is diesel exhaust particulate (DEP). Air pollution has been linked with increased risk of recurrent myocardial infarction (MI) and MI related death (Brook, 2008). This may be due, in part, to effects on atherosclerotic plaque stability and blood clotting tendency. Whether exposure to DEP changes the response of the heart to ischaemia, resulting in increased damage after MI is less well documented. The work described in this thesis was designed to investigate the hypothesis that pulmonary instillation of DEP would increase vulnerability of the heart to subsequent myocardial reperfusion injury secondary to activation of a systemic inflammatory response, endothelial dysfunction and triggering of transient receptor potential vanilloid 1 (TRPV1) mediated autonomic reflexes in the lung.

Examination of bronchoalveolar lavage (BAL) fluid revealed pulmonary inflammation 6 h after exposure to DEP, characterised by neutrophil infiltration, raised levels of the inflammatory mediator interleukin-6 (IL-6) and an increase in alveolar permeability demonstrated by increased levels of protein in the lavage fluid. Pulmonary inflammation was largely resolved 24 h after exposure. While there was no indication of systemic inflammation at 6 h after DEP instillation, the levels of two inflammatory mediators, IL-6 and tumour necrosis factor alpha (TNFα) were increased in the plasma by 24 h after exposure. DEP had no affect on blood flow responses to the endothelium dependent dilator acetylcholine (ACh) in rat hind-limb vasculature *in vivo* at 6 or 24 h. In summary, while exposure of rats to DEP can induce both pulmonary and systemic inflammation, it does not modify endothelium-dependent vasodilatation.

Ischaemia-reperfusion (I/R) was induced *in vivo* in anaesthetised rats and *ex vivo* in buffer perfused hearts from rats that had received DEP *in vivo* 6 h earlier. In both *in vivo* and *ex vivo* I/R models, infarct size (unstained by triphenyltetrazolium choride)

was significantly increased in hearts from DEP-instilled rats relative to hearts from saline-instilled or non-instilled rats. Baseline oxidant stress, determined by electron paramagnetic spin resonance (EPR) in heart perfusate, was also significantly higher in perfusate of hearts from DEP-instilled rats. In summary, a single exposure of the lung to DEP leads to priming of the myocardium for I/R injury.

As the results cited above illustrated, priming of hearts appeared unlikely to be due to either coronary vascular endothelial dysfunction or systemic inflammation. At 6 h post exposure, DEP was associated with increased blood pressure and myocardial hypersensitivity to ischaemia-induced arrhythmias, both suggestive of sympathetic activation. The beta 1 ($\beta_1$) selective blocker metoprolol was used to investigate the role of the sympathetic nervous system (SNS) in transmitting the influence of DEP in the lung to the myocardium via $\beta_1$ adrenoceptor activation. Administration of metoprolol (10 mg/kg, intraperotineal) at the time of DEP instillation into the lung was found to protect the heart from potentiation of *ex vivo* reperfusion injury. Metoprolol was also effective in reducing oxygen free radical generation from these hearts. The TRPV1 antagonist AMG 9810 was also used to study the role of TRPV1 receptors in mediating the priming influence of pulmonary DEP to the myocardium since activation of sensory receptors have been reported to modify sympathetic output via feedback to the central nervous system (Widdicombe *et al.*, 2001). Co-administration of AMG 9810 (30 mg/kg) *in vivo* with DEP into the lung was found to prevent enhancement of *ex vivo* reperfusion injury associated with DEP instillation alone.

Collectively these results have demonstrated that a single exposure of the lung to DEP leads to priming of the myocardium for I/R injury. Furthermore, this priming occurs via activation of a pulmonary sensory reflex that is likely to involve secondary activation of systemic $\beta_1$ adrenoceptors.

# Presentations

## Oral

‘Diesel Exhaust Particulate Pulmonary Exposure And Myocardial Infarction In Rat’ at British Pharmacological Society (BPS) Winter Meeting; London, 2011.

‘Sympathetic activation in vivo mediates enhances myocardial reperfusion injury ex vivo following intra-tracheal instillation of diesel exhaust particulate in rats’ at the Young Life Scientists (YLS) Symposium: Autonomic control in health and disease; Bristol, April 2011.

## Poster

‘Activation of pulmonary sensory receptors following short-term exposure to diesel exhaust particulate primes the heart for increased ischaemia-reperfusion injury’ at the Cardiovascular and Metabolic Disease Signature Program Research Day; Albuquerque, March 2012.

**‘**Tracheal instillation of diesel exhaust particles exacerbates myocardial ischaemia and reperfusion injury in rats’ at the American Heart Association: Basic Cardiovascular Science Conference; New Orleans, July 2011).

‘Sympathetic activation *in vivo* mediates enhanced myocardial reperfusion injury *ex vivo* following intratracheal instillation of diesel particulate in rats’ at the Young Life Scientists (YLS) Symposium: Autonomic control in health and disease; Bristol, April 2011.

‘DEP instillation induces pulmonary & systemic inflammation but no change in *ex vivo* or *in vivo* endothelial function in rats’ at the 14$^{th}$ meeting of the Scottish Cardiovascular Forum (SCF); Aberdeen, February 2011.

‘Does exposure of the lung to diesel exhaust particlulate modify endothelial function in the rat hind limb?’ at the 13th meeting of the Scottish Cardiovascular Forum (SCF); Glasgow, February 2010; and at the Vascular Biology Summer School; Bristol, July 2010.

‘Diesel exhaust particles activate macrophages to stimulate MCP-1 production in endothelial cells’ at the 12th meeting of the SCF; Inverness, January 2009; and the Society for Experimental Medicine (SSEM) scientific meeting; Edinburgh, November 2008.

# Papers

**Diesel exhaust particulate induces pulmonary and systemic inflammation in rats without impairing endothelial function ex vivo or in vivo.**

Robertson S, Gray GA, Duffin R, McLean SG, Shaw CA, Hadoke PW, Newby DE, Miller MR.

Particle and Fibre Toxicology 2012 9(1):9

**Diesel exhaust particulate-exposed macrophages cause marked endothelial cell activation.**

Shaw CA, Robertson S, Duffin R, Tabor CM, Donaldson K, Newby DE, Hadoke PW.

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# Abbreviations

| Abbreviation | Definition |
|---|---|
| AA | arachidonic acid |
| AAR | area at risk |
| AC | adenylyl cyclase |
| ACh | acetylcholine |
| ANKTM1 | ankyrin transmembrane potential 1 |
| ANS | autonomic nervous system |
| APHEX2 | Air pollution and Health: A European Approach |
| ApoE ($^{-/-}$) | apolipoprotein-E |
| ATP | adenosine triphosphate |
| BAL | broncheoalveolar lavage |
| BCA | bicinchoninic acid |
| BH4 | tetrahydrobiopterin |
| BSA | bovine serum albumin |
| $Ca^{2+}$ | calcium |
| CAM | cell adhesion molecule |
| CaMKII | calcium/calmodulin kinase II |
| cAMP | 3', 5' cyclic adenosine monophosphate |
| CAP | concentrated ambient particles |
| CB | carbon black |
| cGMP | cyclic 3', 5' guanosine monophosphate |
| CGRP | calcitonin gene-related peptide |
| CL | chemiluminescence |
| CNS | central nervous system |
| CO | carbon monoxide |
| $CO_2$ | carbon dioxide |
| $CP^{\cdot}$ | 3-carboxy-proxy |

| | |
|---|---|
| CPH | 1-hydroxy-3-carboxy-pyrolidine |
| CPP | coronary perfusion pressure |
| CPZ | capsazepine |
| CRP | C reactive protein |
| DBP | diastolic blood pressure |
| DE | diesel exhaust |
| DEP | diesel exhaust particulate |
| DRG | dorsal root ganglia |
| ECG | electrogardiogram |
| EDHF | endothelium-derived hyperpolarising factor |
| ELISA | enzyme-linked immunosorbent assay |
| eNOS | endothelial nitric oxide synthase |
| ET-1 | endothelin-1 |
| EPR | electron paramagnetic resonance |
| FBF | femoral blood flow |
| FITC | fluorescein isothiocyanate |
| fMLP | formyl-Met-Leu-Phe-OH |
| FS | forward scatter |
| G-CSF | granulocyte colony-stimulating factor |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GSH | glutathione |
| GTN | glyceryl trinitrate |
| GTP | guanosine trisphosphate |
| $H_2SO_4$ | sulphuric acid |
| HBF | hind-limb blood flow |
| H & E | haematoxylin and eosin |
| HR | heart rate |
| HRP | horseradish peroxidase |
| HRV | heart rate variability |
| HUVEC | human umbilical vein endothelial cells |
| ICAM | intracellular cell adhesion molecule |

| | |
|---|---|
| IL | interleukin |
| I/R | ischaemia/reperfusion |
| KO | knockout |
| LAD | left anterior descending |
| LV | left ventricle |
| MAP | mean arterial pressure |
| MFI | median fluorescence intensity |
| MI | myocardial infarction |
| MPMVEC | mouse pulmonary microvascular endothelial cells |
| NAC | N-acetylcysteine |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| NIST | National Institute of Standards and Technology |
| NKκβ | nuclear factor kappa beta |
| NOx | nitric oxides |
| NOS | nitric oxide synthase |
| NTS | nucleus tractus solitarius |
| $O_3$ | ozone |
| PBS | phosphate buffered saline |
| PCI | percutaneous coronary intervention |
| PCR | polymerase chain reaction |
| $PGI_2$ | prostacyclin |
| PM | particulate matter |
| PMN | polymorphonuclear leukocyte |
| RBC | red blood cell |
| ROFA | residual oil fly ash |
| ROS | reactive oxygen species |
| RPP | rate pressure product |
| SBP | systolic blood pressure |
| SH | spontaneously hypertensive |
| SNP | sodium nitroprusside |
| SNS | sympathetic nervous system |

| | |
|---|---|
| SOD | superoxide dismutase |
| SOx | sulphur oxides |
| SP | substance P |
| SPM | synthetic polymer microsphere |
| SS | side scatter |
| TG | trigeminal ganglia |
| TM | transmembrane |
| TMB | 3, 3', 5, 5' tetramethylbenzidine |
| TRP | transient receptor potential |
| TRPC | transient receptor potential classical |
| TRPM | transient receptor potential melastatin |
| TRPML | transient receptor potential mucolipin |
| TRPP | transient receptor potential polycystin |
| TRPV1 | transient receptor potential vanilloid 1 |
| TTC | 2, 3, 5-triphenyltetrazolium chloride |
| UFP | ultrafine particle |
| VCAM | vascular cell adhesion molecule |
| WBC | white blood cell |
| WHO | World Health Organisation |

# Chapter 1

# Introduction

## 1.1 Atmospheric pollution

### 1.1.1 Air pollution and mortality: Historical perspective

The detrimental effects of air pollution were recognised as early as 1872 when the Scottish chemist, Angus Robert Smith wrote a book detailing air pollutants as hazardous components of urban air (Smith RA, 1872). The excess deaths noted during foggy conditions (through industrial and domestic combustion of solid fuels) in the Meuse Valley, Belgium in 1930 (Nemery *et al.*, 2001), Donora, Pennsylvania in 1948 (Helfand *et al.*, 2001) and London in 1952 (Bell *et al.*, 2001; Davis *et al.*, 2002) further increased public awareness of air pollution related health problems. Increased consensus on the health impacts of air pollution saw the enforcement of legislation (such as the United Kingdom Clean Air Act of 1956) focussed on reducing air pollution. Despite these laws having been largely successful in reducing levels of air pollutants, air pollution continues to pose a significant threat to health. The World Health Organisation (WHO) has estimated that the number of deaths attributable to air pollution has risen 16.5% between 2004 and 2008 to 1.34 million deaths per year (WHO Statistics: http://www.who.int/phe/health_topics/outdoorair/databases/burden_disease/en/index.html). Moreover, air pollutants appear to have no demonstrable safe level or threshold for significant adverse health effects (Ware, 2000).

### 1.1.2 Components of atmospheric pollution

Air pollution is a heterogeneous, complex mixture of gases and particulate matter (PM). Gaseous pollutants include carbon monoxide (CO), carbon dioxide ($CO_2$), nitrogen oxides (NOx), ozone ($O_3$) and sulphur oxides (SOx) (Brook *et al.*, 2004). Both gaseous and particulate components have been associated with a spectrum of health effects, including increases in mortality due to cardiovascular and respiratory disease, as well as hospital admissions and ill health related to these diseases (Burnett *et al.*, 1997; Dockery *et al.*, 1993; Miller *et al.*, 2007; Pope *et al.*, 2004; Sunyer *et al.*, 2003). However, as well as in having an independent effect on health,

components of air pollution can interact giving rise to antagonistic, additive or synergistic effects (Chitano *et al.*, 1995). The work described in this thesis focuses on the PM component of air pollution. PM constitutes only 2% of the total mass of air pollution (Brook *et al.*, 2010), yet is considered the major perpetrator of ill health caused by air pollution (Brook, 2008; Mills *et al.*, 2009).

PM itself is a heterogeneous amalgam of solids and liquids with its concentration, size and chemical composition varying both temporally and spatially (Brook *et al.*, 2004). PM originate from a variety of sources, being either man-made (eg, fuel combustion in motor vehicles and industrial sources) or natural (eg, windblown dust, sea salt and forest fires) (Brook *et al.*, 2004; Harrison *et al.*, 2000; Simkhovich *et al.*, 2008). In addition to these primary sources of PM, secondary particulates such as nitrates and sulphates can form as a result of gas-to-particle conversion in the atmosphere (Brook *et al.*, 2004).

PM is often categorised by aerodynamic diameter. The term $PM_{10}$ includes all particles with a diameter less than 10 μm. $PM_{10}$ are also called thoracic particles since they can be inhaled into, and accumulate in, the respiratory system. $PM_{10}$ is commonly classified into three further size groupings: coarse particles ($PM_{2.5}$-$PM_{10}$, diameter 2.5-10 μm), fine particles ($PM_{2.5}$, diameter < 2.5 μm) and ultrafine particles (UFP, diameter < 0.1 μm) (Figure 1.1) (Brook *et al.*, 2004). Current health-based air quality standards for PM are mass-based but other factors such as surface area, deposition within the respiratory system, chemical composition and source may be important for predicting health risks (Brook *et al.*, 2004). Indeed, UFP are believed to pose greater health risks than larger size particles due a higher surface-area-to-mass ratio that permits these particles to carry greater amounts of toxins as well as greater total lung deposition (Oberdorster, 2001). The primary contributors of UFP are combustion products found in motor vehicle exhaust (Cyrys *et al.*, 2003). The work described in this thesis focuses on diesel exhaust particulates (DEP) generated by the combustion process in diesel engines since these engines generate more UFP than petrol engines (Donaldson *et al.*, 2005). Typically, combustion-derived DEP consists of a carbon core onto which different chemical constituents are adsorbed

(Figure 1.2). Components include inorganic compounds (eg, nitrates and sulphates), metals (eg, iron, copper and nickel), organic compounds (eg, polycyclic aromatic hydrocarbons) and biological compounds (eg, endotoxin, cell fragments) (Brook *et al.*, 2004). Acute exposure to DEP in animals increases platelet aggregation and thrombosis formation (Yokota *et al.*, 2004; Yokota *et al.*, 2008a), causes oxidative stress in the myocardium (Yokota *et al.*, 2008b) and increases the risk of ventricular arrhythmias under conditions of reperfusion (Yokota *et al.*, 2004; Yokota *et al.*, 2008a).

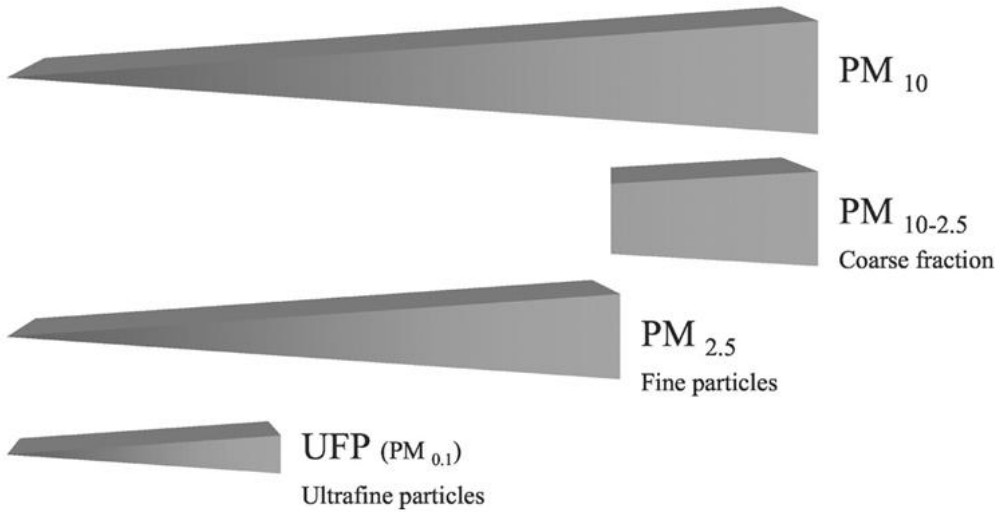


**Figure 1.1 Schematic representation of the aerodynamic size distribution of particulate matter (PM) in air pollution in the range of 0.1 – 10 micrometre (µm). Adapted from (Brook *et al.*, 2004)**

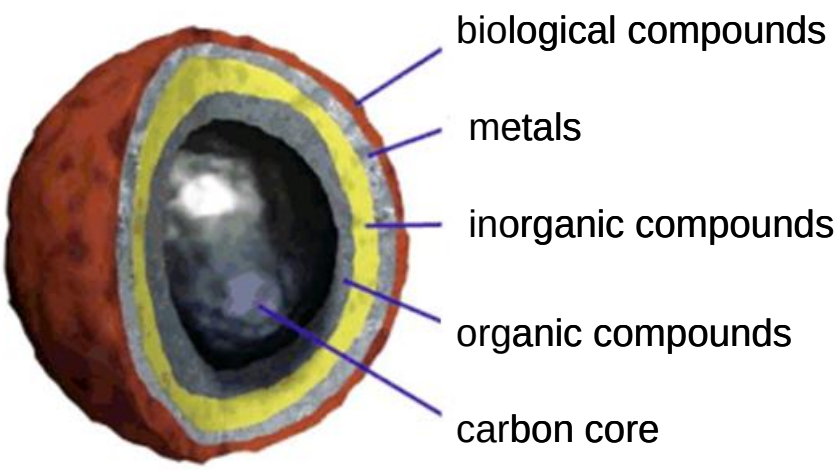


**Figure 1.2 Generalised chemical composition of combustion-derived diesel exhaust particulate (DEP).**

The particles are composed of spherical elemental carbon cores onto which various chemicals and transition metals are attached. Adapted from http://www.caf.us/diesel/dieselhealth/faq.php?site=0

### 1.1.3 PM exposure methods used in the assessment of health effects

A number of *ex vivo* and *in vivo* exposure models have been applied to gain understanding of potential adverse health effects of air pollution. *Ex vivo* models of PM exposure, however, make the assumption that PM can definitely translocate to extra-pulmonary tissues. As will be discussed in more detail in Section 1.3.4, a potential role of PM translocation remains somewhat controversial. The latter is also a limitation of animal studies delivering particles by injection (e.g., intravenous and intraperitoneal). The lung is one of the principal ports of entry for all air pollutants. The two most widely used techniques for the introduction of PM into the lungs of experimental animals are inhalation and intra-tracheal instillation. There are advantages and disadvantages of both delivery methods. The studies described in this thesis delivered DEP to the lungs of rats by intra-tracheal instillation. Instillation was selected, in part, for practical reasons as we are not equipped with inhalation facilities in Edinburgh. Instillation has been used successfully in investigating the health effects of DEP on the cardiovascular system (Madden *et al.*, 2000; Murphy *et al.*, 1998; Yan *et al.*, 2008; Yokota *et al.*, 2004; Yokota *et al.*, 2008a; Yokota *et al.*, 2005; Yokota *et al.*, 2008b). Although intra-tracheal instillation is less physiological than inhalation, it has been widely accepted as a reliable technique to administer particles directly to the respiratory tract in a quantitative and reproducible manner (Driscoll *et al.*, 2000; Miyabara *et al.*, 1998). In comparison to inhalation exposure, instillation results in better dispersion of particles throughout the lobes of rodent lungs and across the alveolar surface (Driscoll *et al.*, 2000; Miyabara *et al.*, 1998). The nasal passages of rodents are more complex than humans and serve as a filter for the inhaled particles. This filtering of particulates conversely reduces the quantity of particles able to reach the alveoli and limits dose reproducibility (Driscoll *et al.*, 2000; Miyabara *et al.*, 1998). Additionally, PM has been reported to induce similar increases in bronchoalveolar lavage (BAL) fluid cell counts for both methods of exposure (Driscoll *et al.*, 2000; Henderson *et al.*, 1995; Miyabara *et al.*, 1998).

## 1.2 Air pollution and cardiovascular disease

### 1.2.1 Epidemiological evidence of the cardiovascular effects of atmospheric pollution

Numerous epidemiological studies conducted worldwide (and summarised in a series of reviews) have in general yielded positive and statistically significant associations between PM exposure and cardiovascular mortality and morbidity (Brook, 2008; Pope *et al.*, 2006a; Simkhovich *et al.*, 2008). Such associations have been reported after short-term and long-term exposure to PM (Brook, 2008; Pope *et al.*, 2006a; Simkhovich *et al.*, 2008). The work described in this thesis focuses on the health effects of short-term exposure to PM. For this reason the major findings from epidemiological studies documenting adverse effects of PM after short-term exposure are discussed here.

Data from a large multi-city study in Europe (APHEA2, Air Pollution and Health: A European Approach) demonstrated a 0.7% increase in acute all-cause mortality in response to a 10 $\mu g/m^3$ increase in $PM_{10}$ (Katsouyanni *et al.*, 2001). Increases in mortality related to short-term PM exposures have been reported in several other well acknowledged single-city and multi-city studies (Dominici *et al.*, 2005; Jerrett *et al.*, 2005; Wietlisbach *et al.*, 1996; Wong *et al.*, 2008).

Cardiovascular events such as myocardial infarction (MI), heart failure, arrhythmia and peripheral arterial disease have all been associated with increased all-cause mortality and hospitalisations after elevated PM (Dominici *et al.*, 2006; Peters *et al.*, 2001a; Peters *et al.*, 2004; Pope *et al.*, 2006b; Sullivan *et al.*, 2005). Indeed, Peters *et al*. (2004) reported that residents of Augsburg had a three-fold increased risk of MI as little as one hour after traffic exposure. Increases in $PM_{2.5}$ concentrations over a 24 hour sampling period have also been reported to increase susceptibility to ventricular arrhythmias among patients with implantable cardioverter defibrillators (Rich *et al.*, 2005). In general, studies have reported stronger exposure-response relationships on cardiovascular outcomes for fine ($PM_{2.5}$) than for coarse ($PM_{10}$)

particles (Brook *et al.*, 2004; Peng *et al.*, 2008). The increase in daily hospital admissions for cardiovascular disease associated with a rise in $PM_{2.5-10}$ levels were lost after adjustment for $PM_{2.5}$ (Peng *et al.*, 2008). The magnitude of risk estimates for the short-term exposure has also been shown to vary depending on the source of pollutant(s), with more consistent or stronger effects observed for traffic-related pollution (Maynard *et al.*, 2007; Peters *et al.*, 2004). Section 1.1.2 addressed the potential toxicity of UFP. However, few epidemiological studies have been conducted for UFP (Delfino *et al.*, 2005; Nel *et al.*, 2006) partly due to the lack of routine monitoring of these particles in the atmosphere. In a review of the epidemiological evidence, specific groups within the population have been identified to be more sensitive to pollutants (Brook *et al.*, 2004). A case-crossover study reported that the risk of death associated with short-term increases in $PM_{10}$ was higher among older individuals (Zeka *et al.*, 2006). Other groups that have been identified to be more sensitive include children, males, those of lower socio-economic status and those with pre-existing medical conditions (Calderon-Garciduenas *et al.*, 2007; Goldberg *et al.*, 2001; Granados-Canal *et al.*, 2005; Laden *et al.*, 2000; Pope *et al.*, 2006b; Zeka *et al.*, 2006).

A number of epidemiological studies have also linked PM exposure with changes in a variety of sub-clinical physiological end-points that relate to cardiovascular health (Simkhovich *et al.*, 2008). PM exposure has been linked to increased levels of various circulating mediators, such as C reactive protein (CRP) (Chuang *et al.*, 2007; Riediker *et al.*, 2004), interleukin-6 (IL-6) (Riediker, 2007; Ruckerl *et al.*, 2007b) and tumour necrosis factor alpha (TNFα) (Calderon-Garciduenas *et al.*, 2008). Indeed, short-term increases in vehicle-derived $PM_{2.5}$ were associated with an increase in serum levels of CRP in nine healthy male North Carolina Highway Patrol troopers (Riediker *et al.*, 2004). PM-related changes in blood coagulation, fibrinolytic factors, blood pressure, vascular reactivity and heart rate variability (HRV; defined as the temporal variation between sequences of consecutive heart beats (Task Force of the European Society of Cardiology 1996)) have also been reported (see review by Simkhovich *et al.*, 2008). Given the burden of epidemiological evidence, a few of the key findings will be highlighted here with

particular emphasis placed on blood pressure and HRV. The general consensus seems to be that PM exposures lead to an elevation of blood pressure (Brook *et al.*, 2009a). Short term exposures to elevated concentrations of $PM_{2.5}$ (mean concentration 10.5 $\mu g/m^3$) among cardiac rehabilitation patients were associated with increases of approximately 2.7 mmHg for both systolic and diastolic blood pressures (Zanobetti *et al.*, 2004). Contrary to this, however, daily changes in PM in Finland, Germany and the Netherlands were not associated with increases in blood pressure in adults with coronary heart disease (Ibald-Mulli *et al.*, 2004). Epidemiological evidence for associations between short-term exposure to PM and cardiac autonomic indexes such as HRV have generally been inconsistent (Peretz *et al.*, 2008a; Peters *et al.*, 2000; Pope *et al.*, 2004; Pope *et al.*, 1999; Riediker *et al.*, 2004; Wheeler *et al.*, 2006). Short-term exposure to PM has been variously shown to decrease (Pope *et al.*, 1999) and to increase (Riediker *et al.*, 2004) HRV in healthy volunteers. Apart from different sources and chemical properties of pollutants, differences in monitoring methods and degree of individual susceptibility may, in part, explain the inconsistencies among findings (Brook *et al.*, 2004). Potential mechanisms for regulation of HRV and autonomic function will be discussed further in Section 1.3.3.

## 1.2.2 Air pollution and vascular function

### 1.2.2.1 Normal vascular function

Vascular endothelial cell injury and dysfunction plays an important role in the pathogenesis of many cardiovascular disorders including atherosclerosis, coronary vasoconstriction and myocardial ischaemia (Celermajer, 1997). Exposure to PM has been associated with a number of adverse cardiovascular health effects (see review by Brook *et al.*, 2004 and Section 1.1). Taken together, these findings could indicate a potential deleterious effect of pollution on normal vascular endothelial cell function. Here an overview is given of normal endothelial cell function. A clear understanding is necessary to better understand the potential effects of PM on aspects of vascular function.

The vascular endothelium, a single cell layer separating blood and vascular smooth muscle, is involved in the regulation of multiple processes, including the control of vascular tone, coagulation, fibrinolysis, inflammation and platelet and leukocyte interactions with the vessel wall (Figure 1.3) (Celermajer, 1997). These multiple responses are mediated by the production and release of various autocrine and paracrine substances (Table 1) in response to physical stimuli, such as shear stress and in response to a variety of endogenous substances, such as bradykinin and thrombin (Celermajer, 1997). The work described in this thesis evaluates vascular relaxant responses to the endothelium-dependent vasodilator acetylcholine (ACh) and the endothelium-independent vasodilator sodium nitroprusside (SNP) in rats following DEP exposure.

ACh increases intracellular calcium ($Ca^{2+}$) concentration by M3 muscarinic receptor (member of the superfamily of G-protein coupled 7 transmembrane (TM) receptors) activation on endothelial cells (Furchgott *et al.*, 1980). The subsequent increase in intracellular $Ca^{2+}$ then stimulates the release of substance(s) (e.g., nitric oxide (NO), prostacyclin ($PGI_2$) and endothelium-derived hyperpolarising factors (EDHFs)) that causes relaxation of the vascular smooth muscle (Figure 1.4) (Furchgott *et al.*, 1980). It should be noted that the contribution of these vasodilators largely depends on the origin of the vessel being analysed. NO is the principal contributor to endothelium-dependent relaxation in conduit vessels (eg, aorta), whereas the contribution of EDHF predominates in smaller resistance vessels (eg, mesenteric vessels) (Graff *et al.*, 2004).

The best characterised EDRF is NO (Palmer *et al.*, 1987). An increase in intracellular $Ca^{2+}$ activates the endothelial constitutive nitric oxide synthase (eNOS), which catalyses the oxidation of L-arginine to L-citrulline and NO (Marletta, 1993). This eNOS-catalysed reaction occurs in two steps and requires the presence of molecular oxygen and a number of co-factors (eg, nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin ($BH_4$)) (Govers *et al.*, 2001). Endothelium-derived NO exerts its smooth muscle vasodilator effect by diffusing to subjacent smooth muscle cells where it primarily activates soluble guanylate cyclase

(sGC) to convert guanosine trisphosphate (GTP) into cyclic 3', 5' guanosine monophosphate (cGMP; Figure 1.4) (Lloyd-Jones *et al.*, 1996). NO donor compounds such as SNP and glyceryl trinitrate (GTN) cause relaxation of the vascular wall, independently of the endothelium, directly releasing NO and thereby activating sGC (Katsuki *et al.*, 1977). The mechanisms by which cGMP causes smooth muscle relaxation include reduction of cytosolic $Ca^{2+}$ and stimulation of myosin light chain dephosphorylation (Collins *et al.*, 1986; Rapoport *et al.*, 1983; Surks, 2007).

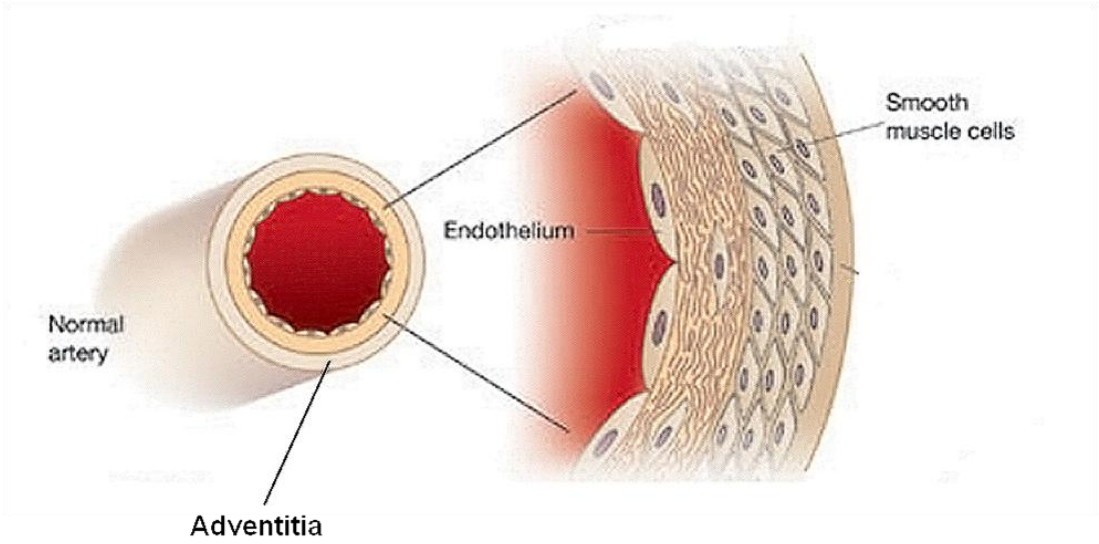


**Figure 1.3 Normal human coronary artery.**

The arterial wall is composed of a single layer of endothelial cells surrounded by supporting smooth muscle. Figure adapted from (Libby, 2002).

| | |
|---|---|
| Vasodilators | NO, prostacyclin, EDHF, bradykinin, adrenomedullin, C-natriuretic peptide |
| Vasoconstrictors | ET-1, angiotensin-II, thromboxane $A_2$, oxidant radicals, prostaglandin $H_2$ |
| Antiproliferative | NO, prostacyclin, transforming growth factor-β, heparin sulphate |
| Proproliferative | ET-1, angiotensin-II, oxidant radicals, platelet-derived growth factor, basic fibroblast growth factor, insulin-like growth factor, interleukins |
| Antithrombotic | NO, prostacyclin, plasminogen activator, protein C, tissue factor inhibitor, von Willebrand factor |
| Prothrombotic | ET-1, oxidant radicals, plasminogen-activator inhibitor-1, thromboxane $A_2$, fibrinogen, tissue factor |
| Inflammatory markers | CAMs (P- and E selectin, ICAM, VCAM), chemokines, nuclear factor κ-B |
| Permeability | Receptor for advanced glycosylation end-products, VEGF |
| Angiogenesis | Vascular endothelial growth factor |

**Table 1.1 Autocrine and Paracrine Substances Released From the Endothelium (Adapted from Verma *et al*., 2002)**

Abbreviations: NO = nitric oxide, EDHF = endothelium-derived hyperpolarising factor, ET-1 = endothelin-1, CAM = cell adhesion molecules, ICAM = intracellular adhesion molecule, VCAM = vascular cell adhesion molecule, VEGF = vascular endothelial growth factor.

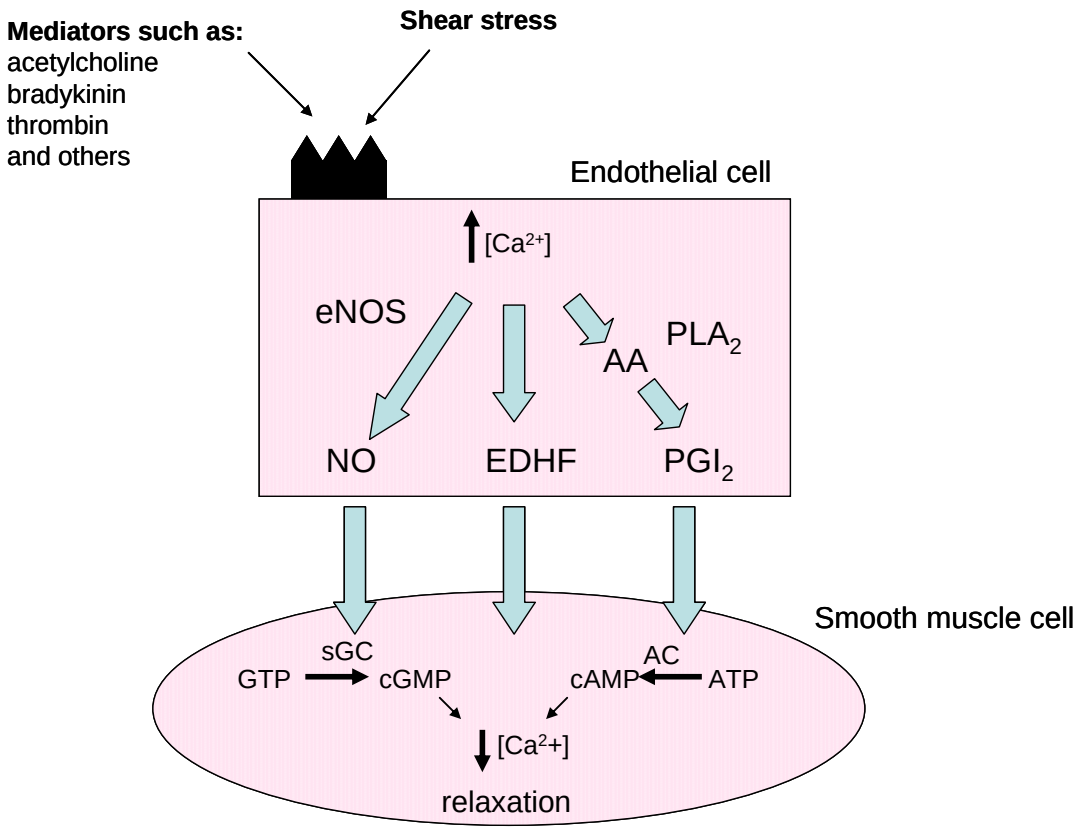


**Figure 1.4 Schematic diagram describing endothelium-dependent relaxation of vascular smooth muscle.**

Endothelial cells respond to various chemical and physical stimuli to cause an increase in intracellular calcium ($Ca^{2+}$) concentrations. The resulting increase in intracellular $Ca^{2+}$ activates endothelial nitric oxide synthase (eNOS) to release nitric oxide (NO). Increased $Ca^{2+}$ also promotes the release of arachidonic acid (AA) by activation of phospholipase $A_2$ ($PLA_2$) as well as several endothelium-derived hyperpolarisation factors (EDHF). The released AA is converted to prostacyclin ($PGI_2$). After release, NO, $PGI_2$ and EDHFs diffuse to the smooth muscle cell and, through different intracellular pathways, decrease cytosolic $Ca^{2+}$ concentrations, resulting in hyperpolarisation and vascular relaxation. NO decreases cytosolic $Ca^{2+}$ via activation of soluble guanylate cyclase (sGC) that synthesises cyclic 3', 5' guanosine monophosphate (cGMP) from guanosine trisphosphate (GTP). In contrast, $PGI_2$ causes relaxation by activating adenylyl cyclase (AC), and stimulating the conversion of adenosine triphosphate (ATP) to 3', 5' cyclic adenosine monophosphate (cAMP). Figure adapted from (Cohen *et al.*, 1995).

#### 1.2.2.2 Pollution and vascular dysfunction

As discussed above, NO released from the endothelium plays an important role in the control of vascular tone. Reduced NO bioavailability therefore decreases the ability of endothelial cells to execute their function in regulating vascular tone and shifts the vasoactive balance towards vasoconstrictive pathways. This section summarises the current literature associating air pollution with reduced NO bioavailability and impaired endothelial cell vasomotor function. Controlled inhalation studies with volunteer subjects revealed impaired endothelium-dependent and –independent dilatation of forearm resistance arteries after short-term exposure to diesel exhaust (DE) (Mills *et al.*, 2005; Tornqvist *et al.*, 2007). However, this is not always the case. In the study by Brook *et al*. (2002), endothelium-dependent vasodilation (induced by ACh) of intact human conduit vessels was unaltered by PM exposure. It is important to note that these clinical studies are generally limited in their ability to investigate the mechanisms underlying endothelial cell dysfunction.

Inhalation of DE in animals has been reported to alter vascular tone *ex vivo* in isolated blood vessels (coronary and mesenteric arteries) in an NO-dependent manner (Cherng *et al.*, 2009; Knuckles *et al.*, 2008). Diminished bioavailability of NO has also been reported after pulmonary exposure to fine PM or nanosized titanium dioxide ($TiO_2$) *ex vivo* in rat skeletal muscle arterioles (Nurkiewicz *et al.*, 2004; Nurkiewicz *et al.*, 2009). Although the precise mechanism of this reduced NO bioavailability remains unclear, several *ex vivo* studies have suggested that functional uncoupling of the NOS protein and increased free radical generation may be important following *ex vivo* (Miller *et al.*, 2009) and *in vivo* (Knuckles *et al.*, 2008; Nurkiewicz *et al.*, 2009) PM exposures. Nurkiewicz *et al*. (2009) detected increased levels of reactive oxygen species (ROS), by hydroethidine oxidation, in isolated rat skeletal muscles after $TiO_2$ nanoparticle exposure *in vivo*. A causative role of ROS was further supported by observations that scavenging ROS *ex vivo* partially restored agonist-mediated dilatation after $TiO_2$ nanoparticle exposure *in vivo* (Nurkiewicz *et al.*, 2009). In other experiments, however anti-oxidant treatment *ex vivo* did not protect against impairment of NO-dependent relaxation induced by PM (Courtois *et*

*al.,* 2008). The same investigators suggested that a systemic inflammatory mechanism may be responsible for the reduced NO-dependent relaxation seen in pulmonary arteries from rats exposed to PM (Courtois *et al.*, 2008). Indeed, it was demonstrated that NO-dependent relaxation could be reversed when treated *ex vivo* with the anti-inflammatory drug dexamethasone (Courtois *et al.*, 2008). Furthermore, the release of TNFα from pulmonary arteries was higher in rats exposed to PM (Courtois *et al.*, 2008). This cytokine has been postulated to be a key player in alterations of the NO signalling pathways in the vasculature (Park *et al.*, 1999). It is important to note that not all studies have reported pulmonary exposure to PM to effect *ex vivo* vasorelaxation responses despite the high doses of particulate employed (Bagate *et al.,* 2004a; Hansen *et al.,* 2007). Similarly negative findings may be under-represented due to the tendency not to report negative data. Nevertheless, overall, there seems to be a general assumption that pulmonary exposure to PM, by either inhalation or instillation, attenuates vascular function *ex vivo*. However, these studies on *ex vivo* vessels remove the vessel from neurohumoural control *in vivo*. To date, studies on air pollutions effects on vasomotion *in vivo* remains uncertain and has yet to be definitively established.

Although this section has focussed on NO, exposure to pollutants is likely to alter the release, turnover or receptor activity of other vasoactive factors such as endothelin-1 (ET-1) to affect reactivity (Batalha *et al.*, 2002; Brook *et al.*, 2002; Cascio *et al.*, 2007; Peretz *et al.*, 2008b). Increased levels of ET-1, a potent vasoconstrictor released from endothelial cells has been associated with alterations in vascular tone due to PM exposure in man (Peretz *et al.*, 2008b) and in animals (Batalha *et al.*, 2002). However, no increase in plasma ET-1 was detected 2 hours after controlled exposures (1 hour with intermittent exercise) of healthy volunteers to dilute DE (Langrish *et al.*, 2009a).

As discussed in Section 1.1.2, diesel combustion exhaust is a major source of atmospheric UFP. To date there have only been a limited number of published studies relating specifically to the effects of DEP exposure on vasomotor function. *In vitro* application of DEP suspension to thoracic aortas from rats has shown loss of

endothelium-dependent vasodilatation (Ikeda *et al.*, 1995; Miller *et al.*, 2009; Muto *et al.*, 1996; Sagai *et al.*, 1993). Excessive production of ROS has been proposed to be responsible for the alterations in vasomotor function due to DEP (Miller *et al.*, 2009; Tzeng *et al.*, 2003). However, these observations are likely to be of limited biological significance as this assumes particles can be translocated from the lung into the circulation and thus directly interact with and influence the vasculature. While studies have demonstrated that translocation of nanoparticles is feasible (Brown *et al.*, 2002; Kreyling *et al.*, 2002; Oberdorster *et al.*, 2004), as discussed later in Section 1.3.4, there remains considerable uncertainty over whether this mechanism underlies the health effects of combustion-derived PM (Brook *et al.*, 2004; Mills *et al.*, 2006). Only one animal study has evaluated endothelial cell vasomotor function following pulmonary exposure to DEP (Hansen *et al.*, 2007). The study found that a single *in vivo* exposure to DEP blunted endothelium-dependent relaxations to agonists in isolated aortic ring preparations from Apolipoprotein-E (ApoE($^{-/-}$)) mice (Hansen *et al.*, 2007). However, wild-type mice were resistant to DEP-induced alterations in vasomotor function. Moreover, there are no pre-clinical studies assessing vasomotor function *in vivo* following pulmonary exposure to DEP.

#### 1.2.2.3 Pollution, plaque formation and thrombogenicity

Epidemiological studies have demonstrated short-term exposures to air pollution to be associated with triggering of MI (see Section 1.2.1). It has been suggested that the pulmonary inflammation induced by exposure to environmental air pollutants may lead to changes in blood coagulation parameters, as well as measures of systemic inflammation. Epidemiological studies have shown an association between short-term increases in air pollution exposure and coagulation promotors such as fibrinogen (Peters *et al.*, 1997; Ruckerl *et al.*, 2007a; Schwartz, 2001), which plays a key role in thrombotic events within atherosclerotic plaques (Koenig, 2003). Circulating levels of fibrinogen were shown to be increased in response to elevated air pollution levels in a large cohort of MI survivors across Europe (Ruckerl *et al.*, 2007a). Animal (Gardner *et al.*, 2000) and human (Ghio *et al.*, 2000) toxicology studies have also reported increases in plasma fibrinogen levels after acute exposure

to air pollutants. Thrombosis formation is a central pathologic mechanism contributing to MI (Libby, 1995). Increased plasma levels of fibrinogen have been associated with an increase in the incidence of ischaemic events (Sweetnam *et al.*, 1996; Thompson *et al.*, 1995). Furthermore, inhalation of DE in humans has been shown to enhance thrombus formation in an *ex vivo* clinical model of arterial injury (Lucking *et al.*, 2008). The enhanced thrombogenicity 6 hours after DE inhalation was suggested to be mediated by platelet-leukocyte activation (Lucking *et al.*, 2008). Tracheal instillation of DEP has also been shown to enhance platelet aggregation and thombosis within 1 hour of exposure in a murine model of vascular injury (Nemmar *et al.*, 2003a; Nemmar *et al.*, 2003b). What is not clear is the mechanism underlying the effects of environmental air pollutants on platelet aggregation. However, Nemmar and co-workers did demonstrate that these pro-thrombotic events were not solely a result of the inflammatory response in the lungs after air pollution (Nemmar *et al.*, 2003b). Pulmonary inflammation and peripheral thrombotic complications were correlated at 6 and 24 hours but not at 1 hour after DEP instillation (Nemmar *et al.*, 2003b).

### 1.2.3 Pollution and blood pressure

Several epidemiological studies have examined associations between short-term exposure to PM and blood pressure (Brook *et al.*, 2009a). The general consensus seems to be that PM exposures lead to an elevation of blood pressure (Brook *et al.*, 2009a). In contrast, controlled human and animal exposure studies evaluating the effects of PM on blood pressure have yielded somewhat inconsistent results (Bartoli *et al.*, 2009b; Cheng *et al.*, 2003; Mills *et al.*, 2008; Mills *et al.*, 2005; Vincent *et al.*, 2001). This may be partly explained by differences in the methodologies of exposure. Vincent *et al*. (2001) reported an increase in blood pressure 2-48 hours after nose-only inhalation exposure to diesel soot in rats. Nemmar and co-workers, on the other hand, assumed that particles could translocate from the lungs into the circulation and demonstrated circulating DEP (24 hours after intravenous injections) to be associated with lower blood pressure in rats (Nemmar *et al.*, 2007). Inconsistencies may also result from using different blood pressure measurement techniques and different PM characteristics and sources.

Investigators have suggested several possible explanations for the positive or negative associations between short-term exposure to PM and blood pressure. Some researchers have suggested that the rapid onset of blood pressure changes with PM exposure might be due to an imbalance in autonomic nervous system (ANS) activity (Bartoli *et al.*, 2009b; Brook *et al.*, 2009b). Healthy volunteers exposed for 3 hours at rest to a controlled concentration of $PM_{2.5}$ showed increased blood pressures and decreased HRV; indices of parasympathetic ANS withdrawal (Brook *et al.*, 2009b). A recent observational study conducted by our clinical team members at the University reported that systolic blood pressure (SBP) was significantly less in healthy subjects wearing a facemask during a 2 hour walk in Beijing (Langrish *et al.*, 2009b). The magnitude of the SBP decrease was strongly associated with increases in HRV, reflecting an autonomic regulation of blood pressure (Langrish *et al.*, 2009b). The role of the ANS is further supported by Bartoli *et al*. (2009) who demonstrated that the raised diastolic blood pressure (DBP) during a 5 hour exposure to concentrated ambient particles (CAP) was significantly attenuated by prazosin, a selective alpha 1 ($\alpha_1$) adrenoceptor antagonist. As will be discussed in more detail in Chapter 1.3.3, PM-evoked activation of sensory nerve endings in the airway lining leads to reflexes involving both the sympathetic and parasympathetic branches of the ANS, lending further support to a neural contribution (Widdicombe *et al.*, 2001). Other studies have suggested that the later onset of blood pressure changes is likely elicited by particle-induced oxidative stress within the lung and/or systemically and subsequent inflammation-induced vascular dysfunction (Brook *et al.*, 2002; Sun *et al.*, 2005; Sun *et al.*, 2008). Lastly, it has also been suggested that alterations in the biological activity of the potent vasoconstrictor peptide ET-1 underlie PM exposure-induced changes in blood pressure (Bouthillier *et al.*, 1998; Calderon-Garciduenas *et al.*, 2007; Peretz *et al.*, 2008b; Vincent *et al.*, 2001).

## 1.2.4 Air pollution and the heart

### 1.2.4.1 Pollution, cardiotoxicity and arrhythmia

As mentioned above, acute exposure to PM has been reported to influence vascular function, blood pressure, coagulation, and thrombosis. All of these events may act as

triggers for a MI. There is also increasing evidence to suggest that short-term exposure to PM is followed by changes in the electrical, mechanical, ionic and energetic state of the myocardium itself.

Epidemiological studies in healthy adults (Riediker *et al.*, 2004) or in subjects with known underlying heart disease (Peters *et al.*, 2000; Rich *et al.*, 2005) have demonstrated that the prevalence of ventricular arrhythmias increases after short-term exposure to PM (see Section 1.2.1). Laboratory studies of PM exposure via inhalation or instillation in animals have generally supported these epidemiological results (Anselme *et al.*, 2007; Farraj *et al.*, 2009; Godleski *et al.*, 2000; Gordon *et al.*, 1998; Muggenburg *et al.*, 2000; Muggenburg *et al.*, 2003; Watkinson *et al.*, 1998; Wellenius *et al.*, 2002). Cardiac arrhythmias after acute exposure to PM have been reported in healthy animals (Muggenburg *et al.*, 2000) but mostly in animal models of vascular and myocardial injury (Godleski *et al.*, 2000; Gordon *et al.*, 1998; Muggenburg *et al.*, 2000; Muggenburg *et al.*, 2003; Watkinson *et al.*, 1998; Wellenius *et al.*, 2002). These investigations reinforce epidemiological studies showing stronger associations between PM and health effects among those with pre-existing diseases (Daigle *et al.*, 2003; Pope, 2000). The key findings of controlled animal exposure studies investigating the acute effects of PM on arrhythmia are summarised in Table 2. Using an anaesthetised model of acute myocardial ischaemia (induced by coronary artery occlusion), Wellenius and colleagues demonstrated that exposure to residual oil fly ash (ROFA) (Wellenius *et al.*, 2002), but not to CAP (Wellenius *et al.*, 2004), increased the frequency of arrhythmias. These changes were associated with decreased HRV, suggesting that particulate-mediated changes in cardiac autonomic balance may be responsible (Wellenius *et al.*, 2002). Decreased HRV has been associated with increased risk for the development of fatal arrhythmias among acute post-MI patients (Bigger *et al.*, 1992; Kleiger *et al.*, 1987). Other studies have, however, shown the pro-arrhythmic effects of air pollutants not to be mediated by changes in HRV (Anselme *et al.*, 2007). Exposure to PM has been associated with systemic inflammation (see Section 1.3.2), which in turn may trigger cardiac arrhythmias (Hatzinikolaou-Kotsakou *et al.*, 2006). Intravenous injections of UFP suspensions from DE engines have also been reported to induce ventricular

premature beats (VPB) in healthy rats *in vivo* (Wold *et al.*, 2006). However, this study assumes the particulate, or its soluble constituents, triggers arrhythmias through direct electrophysiological effects on cardiac myocytes. Whether particles enter the systemic circulation is a matter of intense debate (see Section 1.3.4) and it is currently unknown whether these direct effects on induction of arrhythmia will apply when animals are exposed to DEP via inhalation or instillation. More recently, a study by Hazari *et al*. (2009) reported the potential for a single exposure of ROFA to sensitise the heart to a subsequent dysrhythmic stimulus. 24 hours after exposure, ROFA-exposed spontaneously hypertensive (SH) rats developed arrhythmias at lower doses of aconitine than air exposed SH rats (Hazari *et al.*, 2009). This suggestion that a single exposure to PM may render the heart more sensitive to myocardial injury is discussed further in Section 1.2.4.2.

| Animal | Model | Route of administration | Particle | Dose | Response on arrhythmias | Reference |
|---|---|---|---|---|---|---|
| Dog | Prior coronary occlusion | Inhalation | CAPs | 85-1056 µg/m3 x 6h/d x 3d | Increased | Godleski *et al*., 2000 |
| Dog | Cardiac abnormalities | Inhalation | ROFA | 50 µg/m3 x 3h/d x 3d | No effect | Muggenburg *et al*., 2003 |
| Dog | Healthy | Inhalation | Metals | 3 mg/m3 x 3h/d x 3d | No effect | Muggenburg *et al*., 2000 |
| Rat | Pulmonary hypertension | Instillation | ROFA | 0.25, 1.0, 2.5 mg | Increased | Watkinson *et al*., 1998 |
| Rat | Prior left ventricular MI | Inhalation | ROFA | 3 mg/m3 x 1h | Increased | Wellenius *et al*., 2002 |
| Rat | Pulmonary hypertension | Inhalation | CAPs | >360 µg/m3 x 3h | Increased | Gordon *et al*., 1998 |

**Table 1.2 Overview of animal exposure studies investing the acute effects of particulate exposure on arrhythmia.**

Abbreviations: CAPs = concentrated ambient particles, ROFA = residual oil fly ash, DEP = diesel exhaust particulate, MI = myocardial infarction.

Changes in QT interval and T-wave morphology have been reported both in humans (Henneberger *et al.*, 2005) and in animals (Ghelfi *et al.*, 2008; Godleski *et al.*, 2000) following PM exposure. These parameters, indicative of possible changes in ion channel function, have recently been attributed, in part, to vagal sensory receptors and their reflex effects (Ghelfi *et al.*, 2008; see Section 1.3.3 for a more detailed discussion). Ion channel modulation may account for impaired left ventricular diastolic performance reported in rats following *in vivo* exposure to DEP (Yan *et al.*, 2008). It is important to note, however, that not all animal studies have found PM to affect the heart's electrical conduction system (Muggenburg *et al.*, 2000; Muggenburg *et al.*, 2003). These conflicting results may reflect different sources and chemical properties of pollutants.

As will be discussed in more detail in Section 1.3.1, oxidative stress in the lungs, resulting from exposure to PM, has been shown to induce and/or augment the pulmonary inflammatory response that arises following exposure to air pollution particles. It has also been suggested that this oxidative stress extends beyond the lung. Gurgueira *et al*. (2002) demonstrated for the first time particle-driven increased production of oxidants in the heart *in vivo*. Inhalation of CAPs in rats resulted in 300% increases in the levels of oxidants in the heart, as measured by chemiluminescence (CL) (Gurgueira *et al.*, 2002). There have been some recent attempts to elucidate potential mechanisms linking particle deposition in the lung to oxidative stress in the heart. The increase in oxidative stress in rat hearts 12 hours after intra-tracheal instillation was found to be associated with elevated peripheral neutrophil counts (Yokota *et al.*, 2008b). Other investigators have, however, suggested that particle exposure alters the ANS leading to oxidative stress in the heart (Rhoden *et al.*, 2005). Pre-treatment with either atenolol (beta 1 ($\beta_1$) adrenoceptor antagonist, 5 mg/kg) or glycopyrrolate (muscarinic receptor antagonist, 0.30 mg/kg) abolished the oxidative stress in hearts of rats after inhalation of CAPs (Rhoden *et al.*, 2005). Lastly, increased superoxide production was reported in isolated neonatal rat cardiac myocytes exposed to DEP for up to 24 hours (Okayama

*et al.*, 2006). However, similar to the study by Wold *et al*. (2006), as described above, this study assumes that DEP can be translocated from the lung into the circulation, which remains an unsolved and controversial issue.

#### 1.2.4.2 Pollution and myocardial infarction (MI)

Timely restoration (reperfusion) of blood flow, either by percutaneous coronary intervention (PCI) or fibrinolytic therapy, has been successful in salvaging ischaemic myocardium and improving clinical outcome. Despite this, reperfusion itself may lead to accelerated and additional myocardial injury beyond that generated by ischaemia alone (Yellon *et al.*, 2007). This phenomenon has been termed "reperfusion injury" (Jennings *et al.*, 1960) and may manifest as arrhythmias, contractile dysfunction, endothelial dysfunction, and ultimately cell death (Yellon *et al.*, 2007). The molecular and cellular events involved in myocardial I/R injury are complex, and not fully understood. A pivotal feature of reperfusion is that neutrophils are quickly recruited into the ischaemic myocardium following reperfusion releasing inflammatory mediators (eg, proteases, cytokines and ROS) (Jordan *et al.*, 1999). Mitochondrial damage leading to increasing membrane permeability is also recognised as a contributing factor in the development of I/R injury (Di Lisa *et al.*, 2006).

Exacerbation of reperfusion-induced arrhythmias and increased reperfusion-related mortality has been reported in rats 24-48 hours after intra-tracheal instillation of DEP (Yokota *et al.*, 2004; Yokota *et al.*, 2008a). The same group of investigators also noted that the circulating neutrophil count was significantly elevated among those animals receiving DEP. Furthermore, pre-treatment with superoxide dismutase (SOD) abolished the exacerbation of arrhythmias (Yokota *et al.*, 2004). Based on these observations, the investigators suggested that increased neutrophil infiltration and subsequent ROS generation greatly increases the susceptibility to arrhythmias. Indeed, infiltration and activation of neutrophils into the ischaemic myocardium is

known to play an important role in the genesis of arrhythmias (Jordan *et al.*, 1999). Alternatively, myocardial ischaemia may increase the susceptibility to arrhythmias. In a conscious canine model, CAP inhalation significantly augmented ischaemia-induced electrocardiogram (ECG) changes (ST-segment elevation) compared to the filtered air-exposed control animals (Godleski *et al.*, 2000; Wellenius *et al.*, 2003). A follow-up study implicated impaired myocardial blood flow as contributing to the response (Bartoli *et al.*, 2009a). However, the mechanisms responsible for the observed effects of CAPs on myocardial blood flow have not been identified. Studies conducted by the clinical research department at the University of Edinburgh reported inhalation of dilute DE to exacerbate myocardial ischaemia during exercise in males with stable coronary heart disease (Mills *et al.*, 2007). Only one experimental study has assessed the effects of PM exposure on myocardial ischaemia and reperfusion-induced tissue injury *in vivo*. In that study, the investigators found the infarct size to be larger in mice 24 hours after pulmonary exposure to CAP. However, the mechanism by which pulmonary exposure to pollution is associated with enhanced reperfusion injury is currently unclear.

### 1.2.5 Summary

In summary, considerable research attention has been devoted to short-term increases in particulate levels sparking a cascade of harmful effects on the heart and circulatory system. Reported adverse cardiovascular events include cardiac arrhythmia, ECG changes, blood coagulation and vascular injury. Furthermore, there is some limited evidence to suggest that exposure to PM may render the heart more susceptible to subsequent ischaemic injuries, such as arrhythmias and infarction. However, the mechanism by which pulmonary exposure to pollution is associated with injury in other parts of the body is currently unclear. It is also unknown whether pulmonary exposure to DEP exacerbates ischaemic injury during acute vessel obstruction.

## 1.3 Biological mechanism

Although it is clear that PM has adverse cardiovascular health effects (refer to Sections 1.1 and 1.2), the underlying biological mechanism(s) remain unclear. Proposed biological mechanisms linking PM to cardiovascular disease include: 1) Systemic inflammation as a consequence of "spill-over" of inflammatory mediators (e.g., cytokines) from the lungs to the systemic circulation, 2) ANS imbalance, and 3) Direct toxic cardiovascular effects through the ability of particles (or their associated constituents) to gain direct access into the systemic circulation (Brook *et al.*, 2004). The following section explores each of these in more detail.

### 1.3.1 Pulmonary inflammation

A number of *in vivo* (animal) and *in vitro* (cell culture) studies have shown that various particles (including carbon black (CB), CAP and DEP) can induce lung inflammation (Boland *et al.*, 1999; Chang *et al.*, 2005a; Li *et al.*, 2002; Madden *et al.*, 2000; Nemmar *et al.*, 2003b; Rhoden *et al.*, 2008; Rhoden *et al.*, 2004; Shaw *et al.*, 2009). PM is taken up by phagocytic alveolar macrophages and bronchial epithelial cells *in vitro* and, in turn, up-regulates production of pro-inflammatory cytokines (Fujii *et al.*, 2001; Mukae *et al.*, 2000; van Eeden *et al.*, 2001). Moreover, *in vitro* inflammatory responses to particulates are amplified by interactions between alveolar macrophages and epithelial cells (Fujii *et al.*, 2002). With regard to DEP, incubation of human bronchial epithelial cells with DEP increased the release of the pro-inflammatory cytokines interleukin-8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Boland *et al.*, 1999). An inflammatory response in the airways, characterised by an increased number of neutrophils in sputum, has also been reported after 2 hour exposure of healthy volunteers to inhaled DEP (Nightingale *et al.*, 2000) or whole DE in humans (Salvi *et al.*, 1999).

Several studies have shown PM to induce oxidative stress in alveolar epithelial cells *in vitro* (Baeza-Squiban *et al.*, 1999; Li *et al.*, 2002; Stringer *et al.*, 1998) and in lung

*in vivo (Gurgueira et al., 2002; Rhoden et al., 2004).* For example, rats exposed by inhalation to CAPs increased oxidised lipid levels (indicative of oxidative stress) in the bronchoalveolar lavage (BAL) fluid 24 hours after exposure (Rhoden *et al.*, 2004). ROS may be generated by the particles themselves through their redox ability or released by alveolar macrophages or epithelial cells after particle up take (Donaldson *et al.*, 2003). As mentioned briefly in Section 1.2.4.2, particulate-induced oxidative stress has been reported to mediate some of the inflammatory responses to PM (Becker *et al.*, 1996; Grimm *et al.*, 2010; Kvetnansky *et al.*, 1978; Li *et al.*, 2002; Rhoden *et al.*, 2008; Rhoden *et al.*, 2004). *In vitro* cell culture models have shown exposure to PM to up-regulate activation of redox-sensitive transcription factors (eg, nuclear factor (NF)-κβ) involved in the production of pro-inflammatory cytokines induced upon *in vivo* exposure to PM (Kvetnansky *et al.*, 1978; Shukla *et al.*, 2000). Further supporting ROS as mediators of the effects of particulates, N-acetylcysteine (NAC) pre-treatment prevented the DEP-mediated potentiation of IL-6 release in THP-1 cells (a macrophage cell line) (Li *et al.*, 2002). Pre-treatment with the anti-oxidant NAC also effectively attenuated the influx of neutrophils in BAL fluid from rats after inhalation exposure to CAP (Rhoden *et al.*, 2004). It is important to note that mechanisms other than ROS have been implicated in the regulation of inflammatory responses to PM exposure. Elevated BAL histamine and neutrophil levels have been reported after exposure to DEP in rats (Nemmar *et al.*, 2003a) and humans (Salvi *et al.*, 1999). Moreover, pre-treatment with the histamine receptor antagonist diphenhydramine prevented the increase in neutrophils seen in BAL fluid from DEP-exposed rats (Nemmar *et al.*, 2003a).

### 1.3.2 Systemic inflammation

It has been proposed that PM deposited in the lung may affect the cardiovascular system indirectly through the release of inflammatory mediators from the lung into the systemic circulation (Seaton *et al.*, 1995). Terashima *et al*. (1997) demonstrated that alveolar macrophages, on exposure to inert fine carbon particles, release

inflammatory mediators to stimulate polymorphonuclear leukocytes (PMN) release from the bone marrow, as well as shortening of the transit time of PMN through the marrow. Similar results have been observed in animals exposed to other well-characterised PM (Mukae *et al.*, 2000), as well as in humans during episodes of acute high-level PM exposure (Tan *et al.*, 2000). Moreover, instillation into rabbit lungs of supernatants from alveolar macrophages cultured with PM produced a bone marrow response similar to that produced by instilling the particles themselves (Mukae *et al.*, 2000; Terashima *et al.*, 1997). Significant positive correlations between blood neutrophil count and neutrophil mobilising cytokines (including IL-6 and granulocyte-colony stimulating factor (G-CSF)) levels in BAL fluid 12 hours after the intra-tracheal instillation of DEP in rats further supports the hypothesis that mediators released from the lung contribute to the systemic response (Yokota *et al.*, 2005). Of note, high levels of circulating leukocytes have been associated with an increased mortality (Friedman *et al.*, 1974). This in turn, may be a factor in the cardiovascular mortality that has been associated with air pollution. A recent *in vitro* study in Edinburgh has also supported the potential for airborne particulates to adversely affect the cardiovascular system through indirect pathways (Shaw *et al.*, 2009). In this study, supernatants recovered from DEP-laden monocyte-derived macrophages potentiated pro-inflammatory cytokine release from human umbilical vein endothelial cells (HUVEC) and may serve as a plausible mechanistic link between the previously described association of PM exposure and vascular dysfunction (Shaw *et al.*, 2009).

Increases in circulating levels of CRP, an acute-phase protein produced in the liver, have also been reported after acute exposure to air pollutants both in animals (Lei *et al.*, 2005) and in humans (Peters *et al.*, 2001b; Sandhu *et al.*, 2005). Peters and co-workers showed that elevated serum levels of CRP were positively associated with episodes of increased air pollution in healthy men (Peters *et al.*, 2001b). Increased circulating levels of CRP are associated with systemic inflammation (Rattazzi *et al.*, 2003). In addition, this inflammatory marker may predict the risk of MI among

healthy individuals, as well as among patients with ischaemic heart disease (Berton *et al.*, 2003; Morishima *et al.*, 2002; Ridker *et al.*, 1997). It is important to note, however, that while the pulmonary responses to PM appear to be relatively consistent, results have been notoriously variable regarding systemic inflammation (Mills *et al.*, 2008; Mills *et al.*, 2005; Swiston *et al.*, 2008). Indeed, Mills and colleagues have reported no systemic inflammation in humans following short-term exposure to CAP (Mills *et al.*, 2008) or DE (Mills *et al.*, 2005). Differences in the type and composition of the particulate or co-pollutant may explain these discrepancies.

## 1.3.3 Imbalance of ANS activity

### 1.3.3.1 HRV

The potential role of the ANS in mediating the acute cardiovascular responses to particulate air pollution has already been referred to in previous sections. HRV analysis has been widely used as a non-invasive measurement of cardiac autonomic function (Malik *et al.*, 1990; Pomeranz *et al.*, 1985). Whilst there are some inconsistencies in epidemiological studies (as mentioned in Section 1.2), the general consensus is that HRV is decreased following exposure to PM (Gold *et al.*, 2000; Liao *et al.*, 1999; Pope *et al.*, 1999). Depressed HRV may reflect an increase in sympathetic activation and/or decrease in parasympathetic activation. Low HRV has been associated with increased cardiovascular mortality, even in relatively healthy individuals, and with lowering of the threshold for arrhythmia (Algra *et al.*, 1993; Dekker *et al.*, 2000; Hohnloser *et al.*, 1997; Tsuji *et al.*, 1996). Changes in HRV may therefore to some extent explain the ECG abnormalities and arrhythmias reported in animals and humans after short-term pollution exposures (Godleski *et al.*, 2000; Kodavanti *et al.*, 2001; Mills *et al.*, 2007; Watkinson *et al.*, 1998). It is, however, important to note that in contrast to epidemiological studies, human and animal exposure studies have been less conclusive. While some studies have reported a decreased HRV (Chang *et al.*, 2005b; Devlin *et al.*, 2003; Frampton *et al.*,

2004; Wellenius *et al.*, 2002) after PM exposure, others have reported an increase (Elder *et al.*, 2007; Elder *et al.*, 2004; Gong *et al.*, 2003; Tankersley *et al.*, 2004) or no change in HRV (Fakhri *et al.*, 2009; Muggenburg *et al.*, 2003; Routledge *et al.*, 2006).

#### 1.3.3.2 How does pulmonary exposure to pollution modify ANS activity

To date, how pulmonary PM exposure would modulate autonomic functions remains unclear. This modulation may be a secondary, downstream effect caused indirectly by the release of inflammatory mediators and/or ROS into the systemic circulation (Brook *et al.*, 2004; Rhoden *et al.*, 2005). Rhoden *et al*. (2005) showed that pre-treatment of rats with the anti-oxidant NAC (50 mg/kg i.p.) could prevent PM-dependent changes in heart rate (HR) and HRV. The particles themselves may also modulate cardiac electrophysiology by interacting with ion channels in the myocardium (Brook *et al.*, 2004; Graff *et al.*, 2004). However, it remains in doubt whether particles in the systemic circulation reach a concentration high enough to exert biological effects (see Section 1.3.4). An alternative, and perhaps more likely explanation, is that receptors expressed in airway afferent C-fibres sense pollutants deposited in the respiratory system and, in turn, initiate cardiac autonomic reflexes. Pollutants including, $O_3$ (Lee *et al.*, 2001), sulphur dioxide ($SO_2$) (Wang *et al.*, 1996), wood smoke (Lai *et al.*, 1998) and DE (Wong *et al.*, 2003) have all been shown to stimulate pulmonary C-fibre afferents. What is not clear is the mechanism underlying the activation of these lung sensory receptors in response to air pollution. Data have linked the activation of sensory receptors to the surface charge carried on the PM (Oortgiesen *et al.*, 2000). On the other hand, others have shown activation of sensory receptors in the airways by ROS that are produced in the lung when inflammation accompanies exposure to PM (Bessac *et al.*, 2008). Finally, receptor transactivation or crosstalk between different classes of sensory receptors following PM exposure has also been reported (Skrzypiec-Spring *et al.*, 2007).

#### 1.3.3.2 Transient receptor potential (TRP) channels

The TRP channels are a group of ion channels, located on a large number of tissues and cell types, that often function as cellular sensors to perceive and respond to a wide range of stimuli, including chemical irritants, pain, protons, temperature, mechanical force, taste and osmolarity (Johnson *et al.*, 2006; Moran *et al.*, 2004). This class of ion channel was first described in Drosophila in 1977, where photoreceptors carrying TRP exhibited a transient response to continuous light (Minke, 1977). To date, 28 mammalian TRP channels have been identified and are divided into six sub-families based on amino acid sequence identity: TRPV (Vanilloid), TRPC (Classical), TRPM (Melastatin), TRPML (mucolipin), TRPP (polycystin) and ANKTM1 (ankyrin transmembrane protein 1) (Moran *et al.*, 2004). Most TRP channels are tetrameric calcium-permeable non-selective cation channels with six TM spanning domains, a pore region situated between helices five and six and intracellular carboxy and amino termini (Clapham *et al.*, 2001)(Figure 1.5). Sequence identity across the different sub-families can be as low as 20% (Moran *et al.*, 2004).

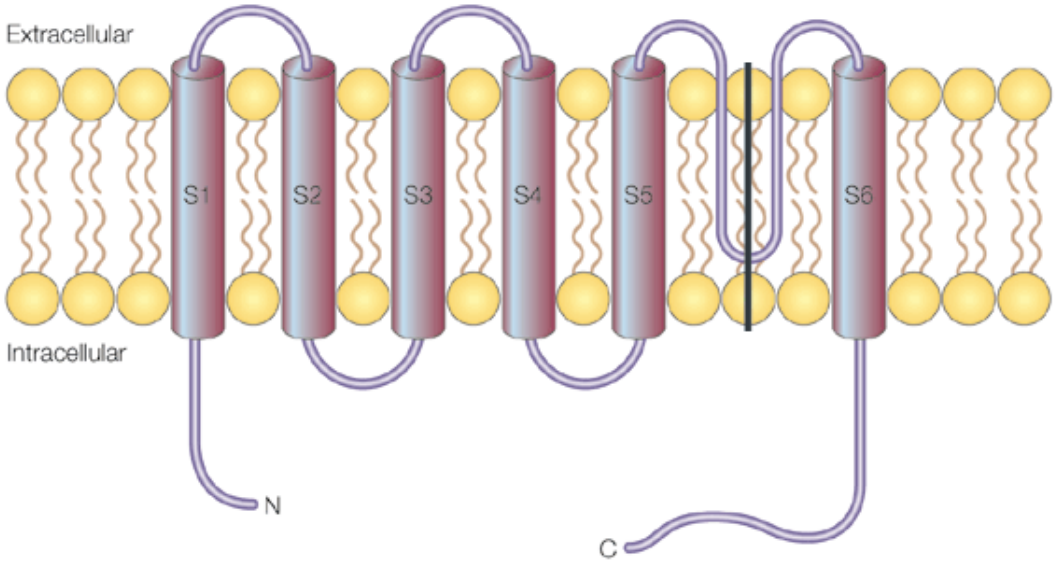


**Figure 1.5 Topological model of transient receptor potential (TRP) channel structure.**

The typical TRP channel consists of six transmembrane domains (TM1-TM6), an intracellular pore loop between TM5 and TM6 and an intracellular carboxy and amino termini. Figure adapted from Clapham *et al.*, 2001.

To date, only one study has reported TRP activation by PM *in vivo* to be damaging to the heart (Ghelfi *et al.*, 2008). These investigators reported that the pharmacological TRPV1 inhibitor capsazepine (CPZ) prevented the cardiac oxidative stress and electrophysiological changes in rats following exposure to CAP (Ghelfi *et al.*, 2008). Chapter 6 evaluates whether pulmonary TRPV1 blockade afects the ability of the hearts from DEP exposed rats to withstand subsequent ischaemic injury. The TRPV1 ion channel was first cloned and characterised in 1997 by functional expression from rat dorsal root ganglia (DRG) tissue on the basis of capsaicin (the pungent component of chilli pepper) sensitivity (Caterina *et al.*, 1997). Besides capsaicin, TRPV1 is activated by noxious heat (<42°C) and, acidic pH, as well as by locally-generated peptides and inflammatory mediators (Caterina *et al.*, 1997; Johnson *et al.*, 2006; Moran *et al.*, 2004). Immunohistochemical studies have localised TRPV1 receptors in non-myelinated (C-fibre) afferents originating from DRG neurons projecting to the airways and lungs (Groneberg *et al.*, 2004; Seki *et al.*, 2006; Watanabe *et al.*, 2005). As discussed above (see Section 1.3.3.1) many different air pollutants have been reported to activate pulmonary C-fibre afferents. Activation of pulmonary C-fibres evokes bursts of action potentials that are conducted to the nucleus tractus solitarius (NTS), in turn leading to reflex changes in autonomic, cardiovascular and respiratory systems (Coleridge *et al.*, 1984) (Figure 1.6).

Vagal afferents can also give rise to an inflammatory airway response (neurogenic inflammation) through local antidromic axon reflexes, releasing neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) (Figure 1.6) (Julius *et al.*, 2001). The released neuropeptides can initiate or amplify inflammation in the airways by acting on both immune (eg, neutrophils and macrophages) and non-immune (eg, epithelial, endothelial and smooth muscle) cells to release inflammatory cytokines (Veronesi *et al.*, 2000). Using a cultured human bronchial epithelial cell line, BEAS-2B, Veronesi and colleagues demonstrated release of IL-6, IL-8 and TNFα cytokine protein 6 hours after exposure to SP or CGRP (Veronesi *et al.*,

1999a). Importantly, TRPV1 has been identified in a range of cell types other than C-fibre sensory neurons, such as airway epithelial cells and mast cells (Biro *et al.*, 1998; Veronesi *et al.*, 2006). Indeed data from numerous *in vitro* studies have shown that TRPV1 activation by capsaicin increases calcium influx and cytokine release in human respiratory epithelial cell lines (Reilly *et al.*, 2003; Seki *et al.*, 2007). *In vivo* studies in rats exposed to capsaicin by inhalation have also reported respiratory inflammation (Reilly *et al.*, 2003). As was discussed in Section 1.3.1, many studies have shown that exposure to inhaled PM induces a pulmonary inflammatory response. Attention has been given to the role of TRPV1 in mediating the inflammatory responses to PM exposure, but the results reported to date have been inconsistent (Oortgiesen *et al.*, 2000; Veronesi *et al.*, 1999b; Witten *et al.*, 2005; Wong *et al.*, 2003). Using a cultured human bronchial epithelial cell line, BEAS-2B release of IL-6 after exposure to ROFA was blocked by the TRPV1 antagonist CPZ (Veronesi *et al.*, 1999b). Involvement of TRPV1 receptors in airway inflammatory responses to PM have also been reported on isolated mouse vagal pulmonary sensory neurons (Oortgiesen *et al.*, 2000). Others have, however, concluded that lung C-fibre activation does not play a dominant role in pollution-induced pulmonary inflammation and injury (Witten *et al.*, 2005; Wong *et al.*, 2003). There has also been little experimental evidence to illustrate the molecular mechanism behind the observed sensitising effect of PM on TRPV1 receptors. Agopyon *et al*. (2003) and others (Oortgiesen *et al.*, 2000) have demonstrated that the effect of PM on TRPV1 is strongly dependent on PM carrying a net-negative surface charge. Neutral synthetic polymer microspheres (SPM) was markedly less effective than their charged counterparts in increasing intracellular $Ca^{2+}$ and IL-6 in the mouse DRG (Oortgiesen *et al.*, 2000). TRPV1 receptors can also be activated by ROS that are produced in the lung when inflammation accompanies exposure to PM (Bessac *et al.*, 2008). In cultured DRG (Story *et al.*, 2003) and trigeminal ganglia (TG) (Jordt *et al.*, 2004) cells, TRPA1 has been shown to co-localise with TRPV1. Moreover, it has been reported that TRPV1 is activated secondary to TRPA1 perhaps due to $Ca^{2+}$ influx or cytokine production (Skrzypiec-Spring *et al.*, 2007).

Consideration of the role of TRPV1 in tissues other than the lung (such as, pancreas, liver and heart (O'Neil *et al.*, 2003; Zahner *et al.*, 2003)), is beyond the scope of this thesis. However, it is worth mentioning that both pharmacological and gene knockout studies have demonstrated activation of TRPV1 receptors on the epicardial surface of the heart to elicit a local cardioprotective feedback loop (Hu *et al.*, 2002; Rang *et al.*, 2004; Wang *et al.*, 2005). For example, Wang and colleagues demonstrated TRPV1 gene deletion to impair post-ischaemic recovery in isolated mouse hearts (Wang *et al.*, 2005).

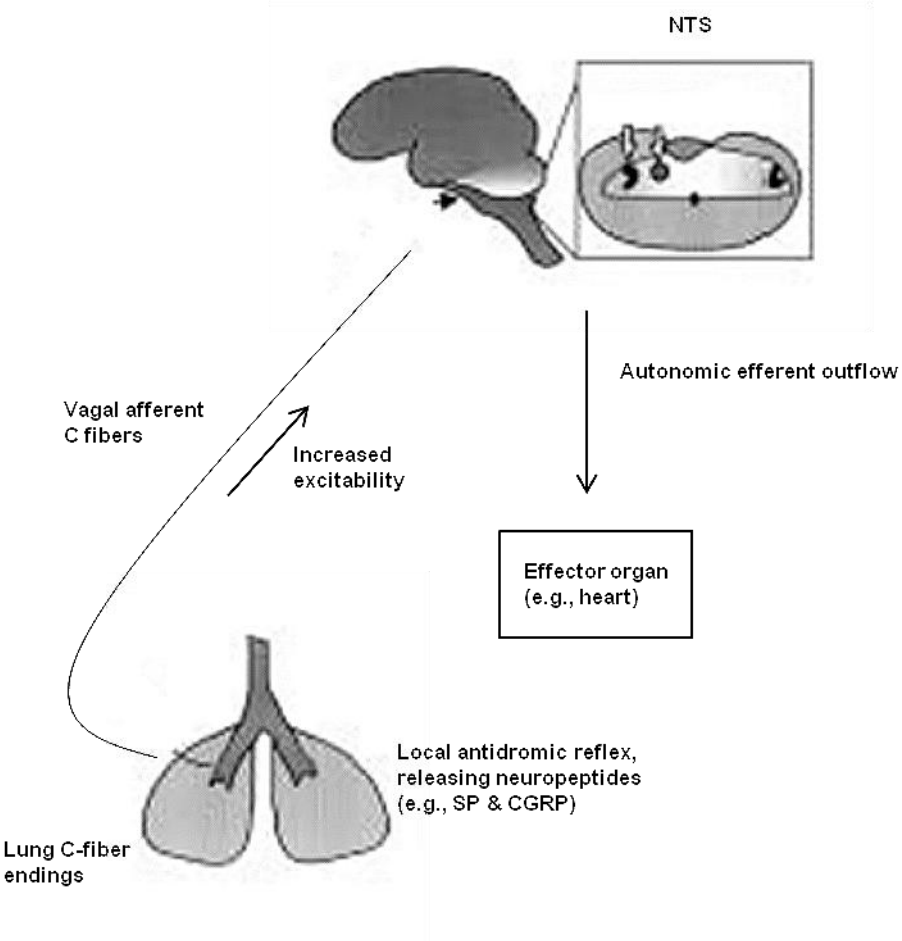


**Figure 1.6 Schematic of central and local axon reflex responses of bronchopulmonary C-fibres.**

A number of exogenous and endogenous stimuli can stimulate C-fibre afferents of the airways. Action potential are generated and transmitted to the nucleus tractus solitarius (NTS), where they are integrated and ultimately influence the autonomic output to effector organs (e.g., heart). Vagal afferents can also give rise to an inflammatory airway response (neurogenic inflammation) through local antidromic axon reflexes, releasing neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP). Figure adapted from Bonham *et al*., 2001.

### 1.3.4 Translocation

Another potential mechanism is that the PM can be translocated from the lung into the circulation and thus exert direct effects on the heart and vessels. Studies in rats with $^{99}$Tc-labelled nanocolloid albumin particles (Nanocoll) by Nemmar *et al*. (2001) found particles to be translocated across the respiratory epithelium to the blood circulation with subsequent uptake in the liver. Other animal studies using inhalation or instillation techniques have reported the translocation of radio-labelled particles from the lungs to extra-pulmonary organs via the blood circulation (Chen *et al.*, 2006; Kreyling *et al.*, 2002; Oberdorster *et al.*, 2002). UFP inhaled and deposited in the lungs has also been reported to enter the central nervous system (CNS) via neuronal pathways (Oberdorster *et al.*, 2004). In theory, the translocation of particles may explain the reported associations between UFP and cardiac events through effects on the ANS and/or initiation of a cascade of reactions involving plaque rupture and thrombosis (Knol *et al.*, 2009). However, the extent of this translocation and its pathological impact is likely to be governed by particle size and surface chemistry (Peters *et al.*, 2006). It is also important to note that the studies cited above, suggesting particle translocation from the lungs detected minute particle translocation ranging from 1% to 6% of the deposited particles (Chen *et al.*, 2006; Kreyling *et al.*, 2002; Nemmar *et al.*, 2001; Oberdorster *et al.*, 2004; Oberdorster *et al.*, 2002). Moreover, of the human studies conducted to date, the majority have detected no or very low levels of UFP outside the lungs (Brown *et al.*, 2002; Mills *et al.*, 2006; Nemmar *et al.*, 2002). DEP translocation has not yet been confirmed. Based on the existing evidence, it is reasonable to assume that if DEP did translocate, it would be unlikely to account for the full biological effects of DEP entering the lung.

### 1.3.5 Summary

Although a number of mechanisms have been proposed to explain the adverse health impact of PM, the issue remains unsolved. Inflammation has been implicated in

many of the actions of DEP and other particles, including actions on the cardiovascular system. However, many other adverse effects have been associated with activation of sensory receptors in the airways leading to alterations in autonomic balance. Furthermore, it has been proposed that the particles may translocate from deposition sites within the lung into the blood circulation with the potential to have direct effects on extrapulmonary organs, but the evidence for this is limited. It is also unlikely that particles would reach high enough concentrations in the circulation to account for the full biological effect.

## 1.4 Hypothesis

The work described in this thesis addressed the hypothesis that:
Intra-tracheal instillation of diesel exhaust particulate (DEP) in rats renders the heart more sensitive to ischaemia/reperfusion (I/R)-induced damage secondary to activation of a systemic inflammatory response, endothelium dysfunction and altered cardiac autonomic reflexes.

### 1.4.1 The specific aims were to:

- Establish the influence of pulmonary exposure to DEP on endothelial cell function *in vivo*, following development of a suitable *in vivo* method for assessing endothelial cell function.
- Establish the extent of pulmonary and systemic inflammation following intra-tracheal instillation of DEP.
- Establish the contribution of *in vivo* neurohormonal activation by pulmonary DEP to myocardial injury following I/R by comparing I/R outcomes *in vivo* with those *ex vivo* in isolated perfused hearts.
- Investigate the mechanisms by which pulmonary exposure to DEP *in vivo* might 'prime' the heart for subsequent injury *ex vivo*, in particular the potential roles of a pulmonary sensory reflex involving activation of transient

receptor potential vanilloid 1 (TRPV1) receptors in the lung and beta 1 ($\beta_1$) adrenoceptors in the systemic circulation.

# Chapter 2

# Materials & Methods

## 2.1 Animals

All experiments were performed according to the guidelines of the Animals (Scientific Procedures) Act 1986 (U.K. Home Office). Licensed procedures were carried out under project licence PPL 60/4247 and personal licence PIL 60/11646. All experiments were approved by the ethical review committee for animal research at the University of Edinburgh. Adult male Wistar rats (250 – 350 g; Charles River, Margate, UK), were housed under controlled environmental conditions (21 ± 2°C; 12-hour light/dark cycle) and received *ad-libitum* access to tap water and to standard laboratory rat chow. All animals were allowed to acclimatise to the environment for at least one week before commencing any surgical or experimental procedures.

## 2.2 Preparation of diesel exhaust particulate (DEP)

Suspensions of DEP (Standard Reference Material (SRM) 2975; National Institute of Standards and Technology (NIST), USA) were prepared in 0.9% sterile saline at a concentration of 1 mg/ml and then sonicated for 5 minutes in an ice bath using a probe-type sonifier (US70; Philip Harris Scientific, Lichfield, U.K.) set at 70% power (5 Hz). The particle size analyser (Brookehaven PS90, Brookhaven Instrument Corporation, New York, USA) estimated the mean diameter of the DEP to be ~ 150 nanometres (nm). Previous data from our laboratory demonstrate DEP suspensions to be endotoxin free (Shaw *et al.*, 2009).

## 2.3 Intra-tracheal instillation protocol

Rats were anaesthetised using 5% isoflurane (Meriol, Essex, UK) delivered in 100% oxygen, and then positioned on an incline board at a 45° angle with the head upwards. With the tongue held out, a paediatric laryngoscope was used to visualise the vocal cords. A 2 mm Luer port cannula (Portex, Kent) was inserted into the trachea and used to administer 0.5 ml DEP suspension (0.5 mg/rat), or an equivalent volume of

0.9% sterile saline, into the lungs. The dose used is comparable to previous studies (Madden *et al.*, 2000; Murphy *et al.*, 1998; Yan *et al.*, 2008; Yokota *et al.*, 2008b) and experiments in this laboratory have shown this dose of DEP to induce systemic effects without acute toxicity (McQueen *et al.*, 2007). Non-instilled rats were included to ensure that the instillation procedure itself had no notable effects on the parameters studied.

## 2.4 Collecting whole blood and plasma

Blood was collected in heparinised syringes (100 $IU.ml^{-1}$ final concentration; Multiparin® CP Pharmaceuticals, Wrexham, UK) via the abdominal aorta (whole blood cell (WBC) counts and measurements of plasma pro-inflammatory cytokine concentrations) or in heparinised tubes (100 $IU.ml^{-1}$ final concentration) after decapitation (measurements of plasma catecholamine concentrations). Whole blood was used immediately for basic haematological parameters (Chapter 2.5.4). Cytokine concentrations (Chapter 2.5.3) were measured in plasma obtained after centrifugation of whole blood at 1500 g for 5 minutes at 4°C. Catecholamine concentrations (Chapter 2.6) were measured in plasma obtained after centrifugation of whole blood at 1000 g for 15 minutes at 4°C. All plasma samples were stored at -80°C until time of analysis.

## 2.5 Assessment of pulmonary and systemic inflammation

### 2.5.1 Bronchoalveolar lavage (BAL)

A BAL was performed to assess pulmonary inflammation 6 and 24 hours after DEP instillation. Briefly, the trachea was exposed and cannulated (0.3 mm outer diameter (o.d.), stainless steel) through which the lungs were lavaged with 8 ml of physiologic saline. The fluid was retrieved by gentle syringe aspiration and this procedure was repeated four times. The last three aspirated lavage fluids were pooled and

immediately placed on ice. The recovered lavages were centrifuged at 2000 x g for 5 minutes. The supernatant from the first lavage was separated into 1 ml aliquots and stored at -80°C until required for biochemical determination. The supernatants fraction from the subsequent lavages were decanted and discarded. All the cells were re-suspended in 1 ml of physiologic saline. The total cell number in the BAL fluid was measured using an automatic cell counter (Sartorius Steadman, Chemometec, Nuclecounter®, Gydevang, Denmark (941-0002)). Cytospin preparations of 10,000 cells in 300 µl phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) were prepared by cytocentrifugation (Shandon cytospin 3 centrifuge) onto glass slides (Superfrost Plus, 75 mm x 25 mm, VWR International Ltd., Leicestershire, UK) for 3 minutes at 300 rpm. After air-drying, the cytospin preparations were fixed in 100% methanol for 1 minute and then stained using Diff-Quik™ physiological stain (Raymond A. Lamb, London, UK). The differential cell count was performed using a hemocytometer (Sigma-Aldrich, Dorset, UK) under 100x magnification. Results were expressed as absolute number of macrophages, neutrophils and other leukocytes. A representative picture of a BAL cytospin slide from a rat 6 hours after the instillation of DEP is shown in Figure 2.1.

### 2.5.2 Bicinchoninic acid (BCA) assay

Increased lung permeability has been associated with airway inflammation (Henderson, 2005). Increased lung permeability is usually accompanied by increased protein leakage into the lungs (Henderson, 2005). Total protein levels in BAL fluid were determined using a standard bicinchoninic acid kit (BCA, Thermo Scientific Pierce, Northumberland, UK) to assess alveolo-capillary permeability as an indicator of lung inflammation. The principle of the BCA method is based on the reduction of copper (II) to copper (I) upon reaction of protein with an alkaline solution. The amount of reduction is proportional to the protein present. Protein concentrations were determined from a standard curve generated using BSA at concentrations from 0.125 to 2 mg/ml. Briefly, the BCA solution and copper (II)

sulphate solution were mixed in a 1:50 volume ratio. 190 µl of the BCA/copper mix was added to 10 µl of the sample or standard and the plates were incubated at 37°C. Duplicates of each standard and sample were assayed. After 30 minutes, the plate was placed in a spectrophotometer set at a wavelength of 570 nm (MRX plate reader. Dynex Technologies; Berlin, Germany).

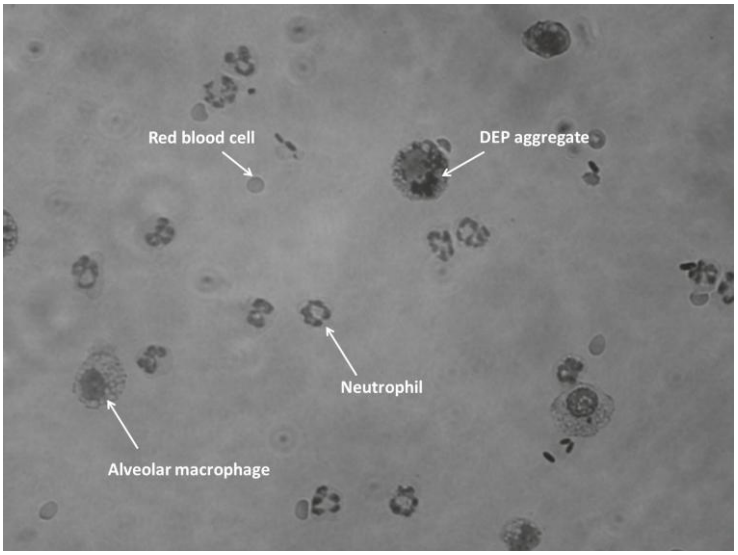


**Figure 2.1 Representative Diff-Quick stained cytospin (x40 magnification) from a rat 6 hours after intra-tracheal instillation of diesel exhaust particulate (DEP).**

Microscopic image showing macrophages (large circular shape and single membrane bound nucleus) and neutrophils (smaller cells with a multi-lobed nucleus).

### 2.5.3 Quantification of cytokines

The concentrations of interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and C reactive protein (CRP) in plasma and BAL fluid were quantified using enzyme-linked immunosorbent assay (ELISA) (R & D Systems, Abingdon, UK) according to the manufacturs instructions. Briefly, ninety-six well flat bottomed plates were coated overnight at room temperature with 100 µl per well of capture antibody (mouse anti-rat IL-6, or mouse anti-rat TNFα, or mouse anti-rat CRP, all from R & D Systems, Abingdon, UK) diluted to a final concentration of 4 µg/ml in PBS. The plates were then washed four times with 300 µl of washing buffer (0.05% Tween 20 in PBS, pH 7.2-7.4, 0.2 µm filtered) and blocked by adding 300 µl of reagent diluent (1%BSA in PBS, pH 7.2-7.4, 0.2 µm filtered) to each well and incubating for a minimum of 1 hour at room temperature. After a further washing step, 100 µl of standards in reagent diluent together with samples and controls were added in duplicate to wells. The plates were incubated for 2 hours at room temperature and then washed with washing buffer. Next, wells were incubated for 2 hours at room temperature with 100 µl of a cytokine-specific detection antibody (biotinylated goat anti-rat IL-6, or biotinylated goat anti-rat TNFα, or biotinylated mouse anti-rat CRP, all from R & D Systems, Abingdon, UK) diluted to a concentration of 4 – 400 ng/ml in reagent diluent. Thereafter, wells were washed and 100 µl of conjugated Streptavidin-horseradish peroxidase (HRP) (diluted 1/200 in reagent diluent) was added to detect the bound biotinylated detection antibody. After 20 minutes at room temperature, wells were washed and 100 µl of the 3, 3', 5, 5' tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldirich, Dorset, UK) was added. The bound conjugated Streptavidin-HRP reacts with the TMB liquid substrate to generate a blue colour directly proportional to the concentration of cytokine present. Plates were incubated in the dark at room temperature and after 20 minutes, the colour development was stopped by adding 50 µl of stop solution containing 2 N sulphuric acid ($H_2SO_4$) to each well.

Plasma interleukin-8 (IL-8) concentrations were quantified using the rat IL-8 ELISA kit (CUSABIO BIOTECH Co, Newark, USA) according to the manufacture's instructions 6 hours after DEP instillation. Briefly, 100 µl of standards in sample diluent together with samples were added in duplicate to a 96 well polystyrene microplate pre-coated with an antibody specific to IL-8. The plates were incubated for 2 hours at 37°C and then the liquid contents carefully removed before adding 100 µl of biotin-antibody (diluted 1/100 in biotin-antibody diluent). After 1 hour at 37°C plates were washed three times with 200 µl wash buffer with a 30 second soak period and then 100 µl of HRP-avidin (diluted 1/100 in HRP-avidin diluent) was added to detect the bound biotinylated antibody. Plates were incubated for 1 hour at 37°C. After a further washing step, 90 µl of the TMB liquid substrate was added. The bound HRP-avidin reacts with the TMB liquid substrate to generate a blue colour directly proportional to the IL-8 concentration present. Plates were incubated in the dark at 37°C and after 20 minutes, the colour development was stopped by adding 50 µl of stop solution to each well.

Following the addition of stop solution, all plates were gently mixed to ensure complete and uniform colour change from blue to yellow. Finally, the plates were read at single wavelength at 450 nm (IL-8) or dual wavelength at 450-540 nm (IL-6, TNFα, and CRP) in a Dynatech MXR microplate reader (Dynatech Laboratories; USA). The lower limits of detection were 31.25, 15.63, 39.06 and 18.75 pg/ml for IL-6, TNFα, CRP and IL-8 respectively. A standard curve was generated for each assay performed of absorbance versus protein concentration. The protein concentration of each sample was interpolated from the standard curve. An example of a standard curve conducted for the IL-6 ELISA is shown (Figure 2.2).

### 2.5.4 Haematological assays

Blood was collected as detailed in Chapter 2.4. The mean values of different haematological parameters; white blood cells (WBC), red blood cells (RBC) and

platelets; were measured with the Coutler®A$^{c}$.T series analyser (Coutler Corporation, Miami, USA).

## 2.6 Plasma corticosterone levels

Plasma corticosterone concentrations were quantified using a commercial ELISA (CUSABIO®BIOTECH, USA) according to the manufacture's instructions 6 hours after DEP instillation. Briefly, 100 µl of standards in sample diluent together with samples (diluted 1/200 in sample diluent) were added in duplicate to a 96 well polystyrene microplate pre-coated with an antibody specific to corticosteroids. The plates were incubated for 2 hours at 37°C and then the liquid contents carefully removed before adding 100 µl of biotin-antibody (diluted 1/100 in biotin-antibody diluent). After 1 hour at 37°C plates were washed three times with 200 µl wash buffer with a 30 second soak period and then 100 µl of HRP-avidin (diluted 1/100 in HRP-avidin diluent) was added to detect the bound biotinylated antibody. Plates were incubated for 1 hour at 37°C. After a further washing step, 90 µl of the TMB liquid substrate was added. The bound HRP-avidin reacts with the TMB liquid substrate to generate a blue colour directly proportional to the corticosterone concentration present. Plates were incubated in the dark at 37°C and after 20 minutes, the colour development was stopped by adding 50 µl of stop solution to each well. The plates were then gently mixed to ensure complete colour change from blue to yellow. Finally, the plate was read at dual wavelength 450/540 nm in a Dynatech MXR microplate reader (Dynatech Laboratories; USA). The lower limit of detection was 6.25 pg/ml. A standard curve was generated of absorbance versus protein concentration. The protein concentration of each sample was interpolated from the standard curve.

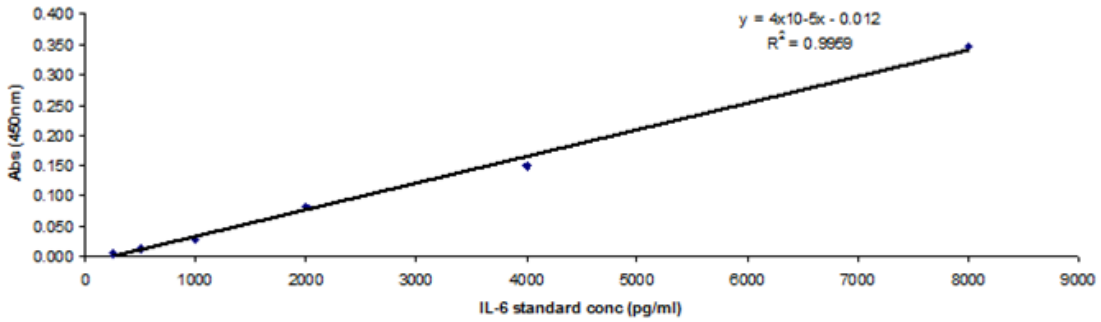


**Figure 2.2 Standard curve for interleukin 6 (IL-6) protein ELISA**

Standard curve for IL-6 ELISA using linear regression analysis. The linear equation between IL-6 protein concentration (x-axis) and absorbance (y-axis) is shown. The R-squared value estimates how well the regression line approximates real data points. R-squared of 1.0 indicates perfect fit.

## 2.7 Myocardial ischaemia/reperfusion (I/R) models

### 2.7.1 *In vivo* induction of ischaemia

The effects of DEP instillation on myocardial damage were investigated using a 45 minute occlusion of the proximal left anterior descending coronary artery followed by reperfusion for 120 minutes as previously described (Jeanes *et al.*, 2008).

Rats were anaesthetised with an intra-peritoneal (ip) injection of sodium pentobarbital (60 $mg.kg^{-1}$ body weight; Sagital, Rhone Merieux, UK) 6 hours after instillation. Animals were placed on a thermostatically-controlled under-blanket (Homeothermic Blanket Control Unit, Harvard Apparatus, UK) to maintain normothermia (37°C).

A small midline incision was made in the ventral surface of the neck and the trachea exposed by blunt dissection of the sternothyroid muscle. A 1.8 mm o.d. stainless steel cannula (Portex, Harvard Apparatus, UK) was then inserted into the trachea through a small transverse incision between the fourth and fifth tracheal rings. The lungs were mechanically ventilated at a rate of 60 strokes/min and a stroke volume of 1.5 ml/100 g body weight (Harvard rodent ventilator model 683; Harvard Apparatus, Kent, UK) to maintain pH, $pO_2$ and $pCO_2$ within physiological limits. The right internal jugular vein was cannulated (0.8mm o.d., 0.5mm inner diameter (i.d.); Portex, Jencons Scientific Ltd, Bedfordhire, UK) for supplemental anesthetic if required.

The left carotid artery was cannulated with polyethylene tubing (0.8mm o.d., 0.5mm i.d.; Portex, Jencons Scientific Ltd) filled with heparinised saline (100 $IU.ml^{-1}$ final concentration; Multiparin® CP Pharmaceuticals, Wrexham, UK). Care was taken to avoid damaging surrounding veins or vagal nerve. The cannula was secured in place

with three 5-0 braided silk ligatures (InterFocus Ltd, England) and connected to a fluid filled pressure transducer (Powerlab/4SP, ADInstruments, UK) for continuous monitoring of the arterial blood pressure (systolic, diastolic and mean) and heart rate. A representative recording trace of arterial blood pressure is shown below (Figure 2.3). Electrocardiograms (ECG) were continuously monitored using subcutaneous wires in a three lead configuration (right arm, left leg, and right leg leads). ECG signals were generated by connecting the lead wires to a Powerlab (Powerlab/4SP, ADInstruments, UK) via a Bridge Amplifier. All data were collected on a MacLab/4e data acquisition system (AD instruments, Sussex, UK) and analysed using Chart™ software (AD Instruments, Sussex, UK) on a Macintosh computer (Power PC G3).

An incision (~2 cm) was then made between the left $4^{th}$ and $5^{th}$ rib and dissection was continued down through the intercostal muscles and the ribs were gently spread with an adjustable screw retractor to expose the heart. To prevent lung injury during surgery, the left lung was temporarily collapsed by placing a saline-soaked gauze swab over the lung and gently pressed away from the heart. The pericardial sac was then opened and the heart was exteriorised by gently lifting with a cotton bud placed on the underside. A piece of 5-0 braised silk suture (11-mm round bodied, InterFocus Ltd) attached to a needle holder was placed around the left anterior descending (LAD) coronary artery. The suture was then threaded through a polyethylene tube and the heart was immediately replaced in the chest cavity. After a stabilisation period of 10 minutes, the coronary artery was occluded by tightening the ligature using the polyethylene tube and clamping in place. Occlusion was verified by the appearance of epicardial cyanosis and a transient drop in arterial blood pressure. Experiments where the procedure produced a sustained decrease in blood pressure of less than 60 mmHg were discontinued. After a 45 minute period of coronary occlusion, reperfusion was initiated by gently releasing the clamp and then monitored for 2 hours under continued anaesthesia. All cardiovascular parameters

were monitored continuously, noting the responses every 15 minutes during the ischemic-reperfusion period. At the end of the 2 hour reperfusion period, the LAD coronary artery was re-occluded and Evans blue (1% w/v in 1 ml PBS) was injected into the right jugular vein catheter to differentiate between the ischaemic area at risk (AAR) and non-ischaemic heart tissue (Figure 2.4). The heart was then rapidly removed, rinsed with PBS and stored at -20°C until analysis.

#### 2.7.1.1 Quantification

The mean arterial pressure was computed as diastolic pressure plus one third of the difference of systolic blood pressure (SBP) minus diastolic blood pressure (DBP). Heart rate (HR) was measured as the period of time between successive systolic peaks. At each time point, all haemodynamic parameters were calculated as the average of five-to-ten successive measurements.

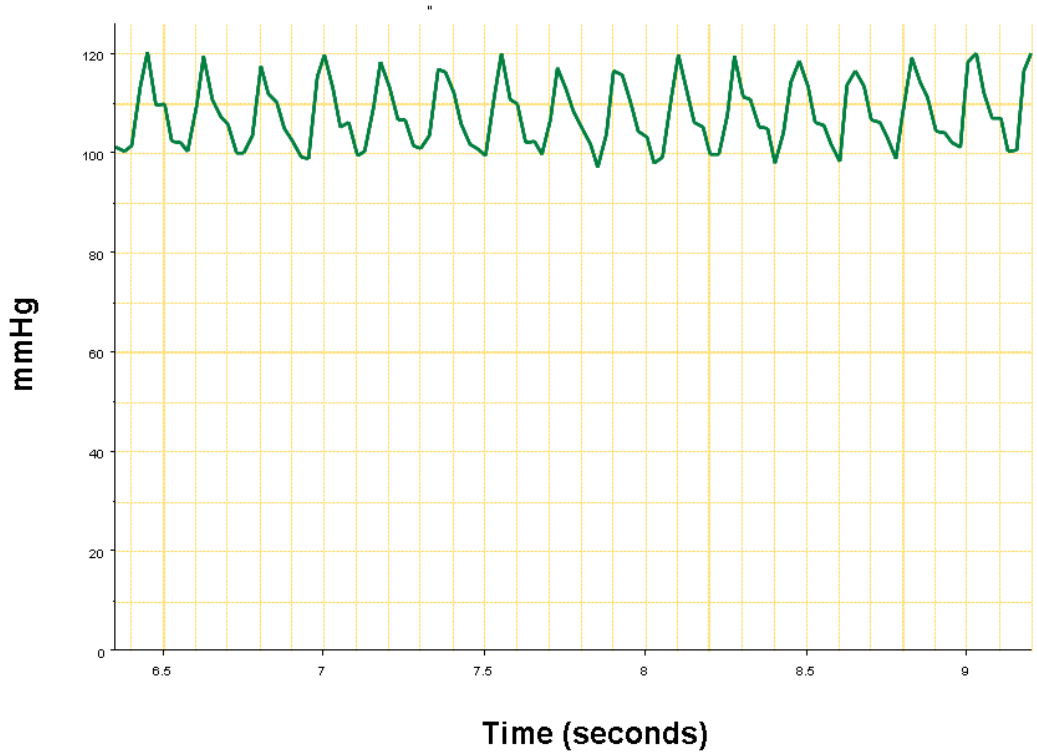


**Figure 2.3 Typical arterial blood pressure waveform recorded from the carotid artery of anesthetised non-instilled rats before the induction of cardiac ischaemia.**

The trace displays arterial pressure in millimeters of mercury (mmHg) versus time in seconds (s) recorded from a pressure transducer connected to the left carotid artery. The peak pressure is defined as the systolic pressure and the minimum pressure is defined to as the diastolic pressure.

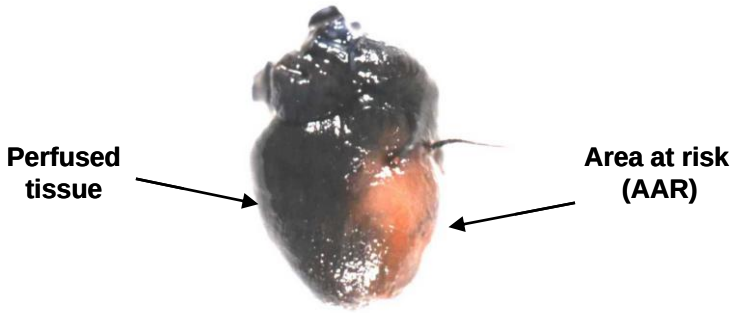


**Figure 2.4 Isolated rat heart showing area at risk of cardiac ischaemia.**

Representative heart collected after 45 minutes of cardiac ischaemia and 2 hours of reperfusion. Evans blue (1% w/v in 1 ml PBS) was injected as a bolus into the jugular vein. The area left unstained by Evans blue was defined as the area at risk (AAR), and the blue stained region was the well-perfused (non-ischaemic) area.

### 2.7.2 Induction of ischaemia *ex vivo* (Langendorff)

The effect of particle instillation on myocardial damage was also investigated *ex vivo* using a Langendorff perfusion system (Figure 2.5). Animals were anaesthetised by inhalation of 5% isoflurane delivered in 100% oxygen. The hearts were removed and immediately arrested in ice-cold HEPES buffer (composition: 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 11 mM glucose 11.1; pH 7.4; 4°C). Immediately before anaesthesia animals received an ip injection of heparin (100 $IU.ml^{-1}$ final concentration; Multiparin® CP Pharmaceuticals, Wrexham, UK) to prevent the formation of thrombi in the excised heart. Following removal of excess connective tissue, the aorta was held below the cannula for a few seconds allowing it to fill with Tyrodes solution (composition: 116 mM NaCl, 20 mM $NaHCO_3$, 0.4 mM $Na_2HPO_4$, 1 mM $MgSO_4$, 4 mM KCl, 11 mM glucose, 2.5 mM $CaCl_2$; 37 ± 1°C), making sure all air bubbles were removed. The cannula was then inserted into the ascending aorta, secured in place and perfused at a constant flow of 10 ml/min (delivered via a ML172 Minipuls 3 Peristaltic pump, AD Instruments, Sussex, UK) for the duration of the experiment. The heated glass chamber of the Langendorff apparatus was placed around the heart in order to provide a warm (37°C) and humid environment. Coronary perfusion pressure (CPP) was measured using a fluid-filled pressure transducer (Powerlab/4SP) attached to a side port immediately above the level of the heart. Following a stabilisation period of 10 minutes, a piece of 5-0 braised silk suture was placed around the LAD coronary artery and ischaemia was initiated using the threaded polyethylene tube as described for *in vivo* experiments (Chapter 2.6.1.1). After 30 minutes of ischaemia, the clamp was released to allow reperfusion for 2 hours. At the end of the 2 hour reperfusion period, the LAD coronary artery was re-occluded and Evans blue (1% w/v in 1 ml PBS) was injected into the aortic cannula to differentiate between the ischemic (AAR) and non-ischemic heart tissue. The heart was then rapidly removed, rinsed with PBS and stored at -20°C until analysis.

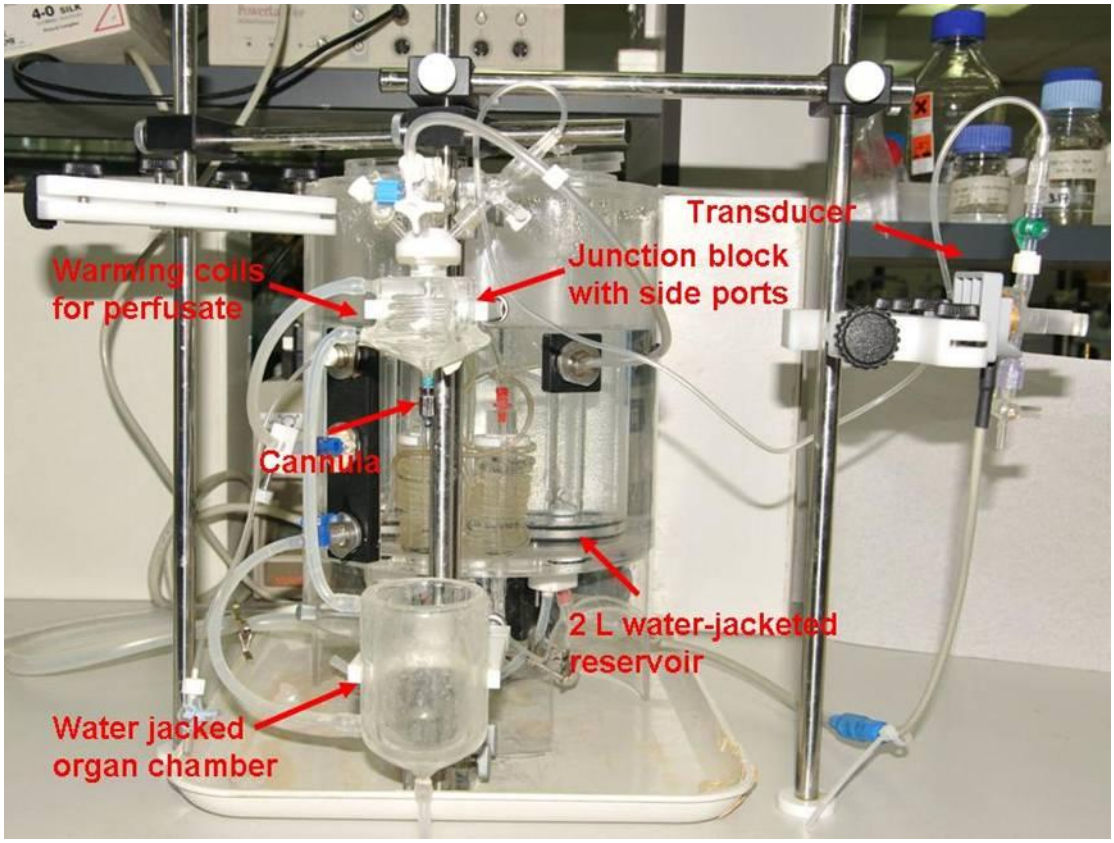


**Figure 2.5 Photograph showing the components of the Langendorff heart preparation.**

Hearts are attached to the Langendorff perfusion system through a cannula in the ascending aorta. Perfusate buffer is pumped through a water jacketed coil. This fluid is then delivered in a retrograde direction down the aorta at a constant flow rate (delivered via a Peristaltic pump). A side port in the junction block is used to connect to a pressure transducer for measurement of coronary perfusion pressure (CPP).

## 2.8 Tissue sampling

Hearts were removed from rats 6 hours after DEP instillation and placed immediately in 0.9% saline. After thoroughly rinsing in 0.9% saline, the atria, large vessels and, connective tissue were trimmed and the heart was cut either longitudinally or transversely, approximately half way between the apex and base to expose the inside of the left and right ventricles. The hearts were fixed for 24 hours in 10% neutral buffered formalin prior to further processing and paraffin wax embedding. The first step of processing involves the removal of water from the tissue by immersion in increasing concentrations of ethanol. Next, as ethanol is not miscible with the paraffin wax, the ethanol from the tissue is removed prior to wax impregnation. This is achieved by transfer of the tissue to a wax miscible agent, also referred to as a clearing agent. Finally, the tissue is embedded in paraffin wax to form tissue blocks. The blocks were stored at room temperature until ready for sectioning.

## 2.9 Histology

### 2.9.1 Triphenyltetrazolium chloride staining

Infarct size was determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, Dorset, UK) staining as previously described by Jeanes *et al*. (2006) with minor modifications. The heart was sliced into 2-3 mm thick transverse sections from apex to base. The slices was then immersed in a 1% w/v solution of TTC in saline (0.9% w/v) at 37°C and stained for 30 minutes. This technique differentiates between viable (viable ischaemic tissue) and infarcted (necrotic ischaemic tissue) tissue within the AAR as identified previously by the absence of Evans blue staining. TTC is reduced to TTC-formazan, an insoluble brick-red precipitate, by dehydrogenase enzymes present in viable myocardial tissue. Thus, significantly lower levels of dehydrogenase enzymes in infarcted myocardial tissue leaves this

tissue unstained (white) by TTC. A representative Evan's blue/TTC-stained section of a rat heart is shown in Figure 2.6. After TTC staining, the slices were immersed for 10 minutes in 10% formalin (Sigma-Aldrich, Dorset, UK) in PBS to enhance the contrast of the stain and then rinsed for 10 minutes in saline (0.9% w/v). Total left ventricle (LV), AAR and infarct were separated by careful dissection and weighed. The AAR was expressed as a percentage of the LV and infarct size as a percentage of either LV or the AAR.

### 2.9.2 Haematoxylin and eosin (H & E) staining

Paraffin wax-embedded hearts were sectioned at 5 μm using a retractable rotary microtome (Leitz Weltslar 1512 microtome) and floated onto a water bath maintained within 40-45°C. After a few minutes to allow any wrinkles to smooth out, the sections were mounted on SuperFrost® Plus glass slides (VH Bio, Tyne and Wear). The slides were then left to air-dry for a minimum of 10 minutes before being placed in a humid chamber at a temperature of 37°C overnight.

For H & E staining, the sections were completely de-waxed by placing in xylene for 1 minute (2 changes of 30 seconds). After de-waxing, sections were re-hydrated through descending grades of alcohol (100% ethanol for 1 minute, 74% ethanol for 1 minute twice) to running tap water. Sections were then placed in Harris haematoxylin (Shandon) to stain cell nuclei dark-blue. After incubation for approximately 5 minutes, the sections were gently rinsed with running tap water (1 minute) and then immersed in alkaline tap water (2-3 drops of ammonia in 400 ml tap water for 1 minute) to stop the reaction. Sections were differentiated in acid alcohol (1% hydrochloric acid in 74% ethanol) for 5 seconds and then immediately washed well with running tap water. Sections were then counterstained in alcoholic Eosin Y (Sigma) for 30 seconds followed by a wash in running tap water and dehydration through a graded series of alcohol (74% ethanol for 1 minute twice, 100% ethanol for 1 minute). Finally, sections were cleared in xylene (2 changes of 2

minutes) and a cover slipped was mounted using DPX mounting media. Neutrophils were viewed with an Image Pro6.2, Stereologer Analyser 6 MediaCybernetics (Buckinghamshire, UK). For quantification, sections were tiled at x100 magnification (using a 100x objective). The entire LV was identified as the area of interest using the Image Pro6.2 computer programme after image acquisition. Neutrophils were identified by their distinct morphological nature showing multi-lobed nuclei at x100 magnification. Cells were counted in the LV from 15 randomly selected areas per tissue section. One tissue section per rat heart was counted.

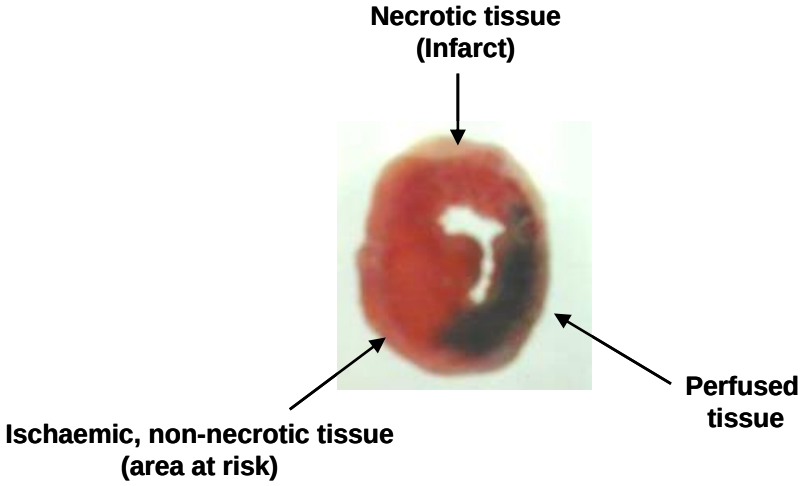


**Figure 2.6 Section of an infracted heart after ischaemia and reperfusion.**

Representative photograph of heart section stained with Evans blue and 2, 3, 5-triphenyltetrazolium chloride (TTC). Tissue stained blue by Evans blue represents normal perfused myocardium. TTC stains the ischemic but viable tissue brick red whereas the necrotic ischemic tissue (infarct) is unstained (white) by TTC.

## 2.10 Assessment of neutrophil activation status by flow cytometry

### 2.10.1 Flow cytometry

Whole blood flow cytometry was used to assess granulocyte activation status following DEP exposure by quantifying the expression of the two cell surface molecules CD11b and L-selectin (CD62L) (Cozzi *et al.*, 2006). Flow cytometry is a method routinely used to identify cell populations based on light scatter and fluorescence. Light scattered by the cells is measured in the forward and sideways directions. Forward scatter (FS) is a measure of cell size, whereas side scatter (SS) is a measure of cell granularity (Ibrahim *et al.*, 2007). When activated, cells show an increase in CD11b expression and a decrease in expression of L-selectin. We hypothesised that exposure to DEP would induce activation of neutrophils. Primed neutrophils would be more likely to adhere to the endothelium causing increased damage during myocardial I/R. Initial attempts at staining of neutrophils with a neutrophil specific antibody (phycoerythrin (PE)-conjugated mouse anti-rat IgG2a mAb, clone RP-1, BD Pharmingen, Oxford, UK) were unsuccessful. Many attempts were made to develop and optimise the protocol including testing various blood anti-coagulants as well as checking for variation between antibody batches. Therefore the expression of CD11b and L-selectin were assessed on all granulocytes (neutrophils, basophils and eosinophils).

Whole blood was withdrawn into a heparin (100 U/ml final concentration, Multiparin®, CP Pharmaceuticals, Wrexham, UK) rinsed 10 ml aspiration syringe and immediately placed on ice. As a positive control a subset of blood samples were stimulated with formyl-Met-Leu-Phe-OH (fMLP) which has previously been shown to increase leukocyte adhesion *ex vivo* (Shen *et al.*, 1999). To determine the optimal concentration of fMLP, heparinised whole blood was treated with increasing

concentrations (0.001 – 10 µg/ml) for 30 minutes at 37°C with gentle shaking. 1 µg/ml was used for all subsequent studies. After 30 minutes, non- and fMLP-stimulated blood was placed on ice for 15 minutes and then stained with saturating concentrations of fluorochrome-conjugated antibodies directed against CD11b (Fluorescein Isothiocyanate (FITC)-conjugated mouse anti-rat mAb, clone OX-42, 10 µg/ml, AbD serotec, Oxford, UK) or CD62L (FITC-conjugated mouse anti-rat, clone OX-85, 0.1 mg/ml, AbD serotec, Oxford, UK). All antibodies were titrated in preliminary experiments to determine the optimum antibody concentration. Following incubation in the dark at room temperature for 20 minutes, erythrocytes were lysed by adding 2 ml of AbD Serotec's Erythrolyse diluted 1:10 in distilled water. After 10 minutes, samples were centrifuged for 5 minutes at 400 g and the supernatant discarded. Cells were then washed in 2 ml PBS containing 1% BSA and centrifuged for 5 minutes at 400 g. Finally, the supernatant was decanted and the cell pellet re-suspended in 200 µl of PBS containing 1% BSA. Unstained cells were also included as a negative control to detect any autofluorescence or background staining. FITC-conjugated mouse IgG2a mAb (10 µg/ml, AbD serotec, Oxford, UK) was used as an isotype control for the test antibodies to estimate non-specific binding.

### 2.10.2 Acquisition and analysis

Fluorescence was detected in the FL-1 channel. The granulocytes were identified and gated based on their distinct forward- and sideways-scatter profiles. Data were presented as the median fluorescence intensity (MFI) for each peak after deduction of the isotype control. All data were collected on a FACS-Calibur (BD Pharmingen, Oxford, UK) flow cytometer and analysis performed using FlowJo Data Analysis Software. Five thousand events were collected from each single sample. A forward versus side scatter dot plot of lysed whole rat blood is shown in Figure 2.7. MFI values of representative histograms are also shown (Figure 2.8).

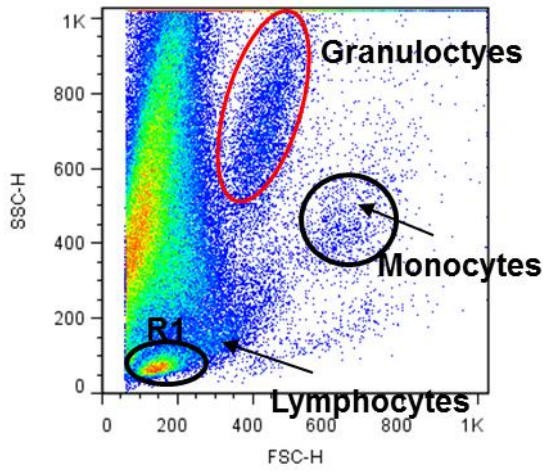


**Figure 2.7 Representative dot plot of forward scatter (FSC-H) versus side scatter (SSC-H).**

Region 1 (R1) is expected to contain cellular debris and dead cells. Lymphocyte, monocyte and granulocyte populations were identified on bases of their forward and sideward scatter properties. A gate (red circular box) was set around the granulocyte population and analysed for CD11b and CD62L expression.

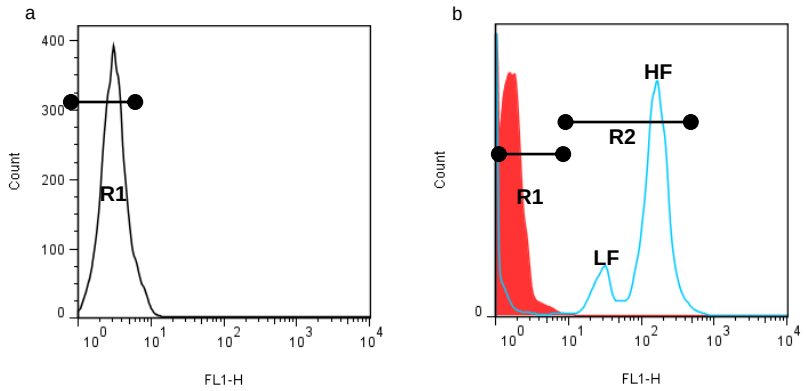


**Figure 2.8 Fluorescence intensity histograms of both stained and unstained granulocytes.**

(a) Fluorescence histogram of unstained granulocytes collected 6 hours following instillation of saline. The peak within the first log decade of the x-axis corresponds to unstained control cells. (b) Fluorescence histogram of CD11b stained granulocytes collected 6 hours following instillation of saline. CD11b antibody showed two fluorescence peaks in the histogram with low (LF) and high fluorescence (HF). Histogram overlay indicates istoype matched control antibody (IgG2a, red histogram). The isotype control showed a similar pattern to unstained cells. Cells within region 1 (R1) are negative for CD11b protein; cells within region 2 (R2) are positive.

## 2.11 Electron paramagnetic resonance (EPR)

Hearts from rats pre-treated 6 hours earlier with intra-tracheal instillation of DEP or saline were isolated and mounted on a Langendorff perfusion apparatus with constant flow as described (Chapter 2.7.2). Oxygen free radical generation from the myocardium was assessed in perfusate using spin-label electron paramagnetic resonance (EPR) (Jeanes *et al.*, 2008; Miller *et al.*, 2009). Chemical spin-traps are molecules that react with highly unstable primary radical species to form a more stable secondary radical that can be detected by EPR. Coronary effluent was collected in 1 ml aliquots (9 drops) into tubes containing the spin trap 1-hydroxy-3-carboxy-pyrolidine (CP-H; Axxora Ltd, Nottingham, UK) to achieve a final concentration of $10^{-3}$M. CP-H has previously been demonstrated to detect oxygen-centred free radicals (Dikalov *et al.*, 1997). Upon reaction with oxygen-centred radicals, CP-H is oxidised to 3-carboxy-proxy (CP$^{\cdot}$; Figure 2.9). The stable radical gives rise to a characteristic 3-line EPR spectrum that can be quantitatively assessed. Pyrogallol was used as a positive control to generate superoxide radicals (Taylor *et al.*, 2004). Hearts were subjected to 30 minutes of coronary occlusion followed by 2 hour reperfusion as described previously (Chapter 2.7.2). Perfusion effluent was collected at baseline, after 0 and 30 minutes of ischaemia and after 0, 1 and 2 hours of reperfusion. All samples were incubated at 37°C for 5 minutes. 50 µl of the sample solution was then drawn up into a glass capillary tube (Scientific Laboratory Ltd, Coatbridge, UK), sealed with claylike sealant (Cristaseal, VWR International, Lutterworth, UK) and placed in an X-band EPR spectrometer (Magnettech MS-200, Berlin, Germany). The EPR spectrum settings were as follows: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1500 mG; center field, 3365 G; sweep width, 43.95 G; sweep time, 30 sec; number of passes, 1. Relative radical concentrations (in arbitrary units, AU) were recorded as the signal intensity obtained from the peak-to-peak height of the first derivative EPR spectrum.

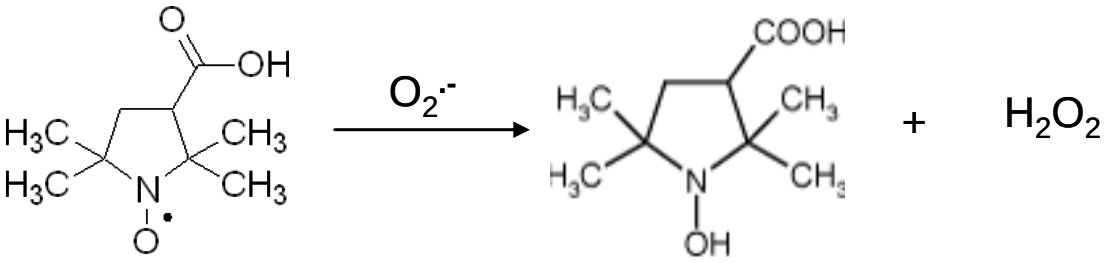


**Figure 2.9 Schematic illustration of the concomitant oxidation of spin-trap CP-H to CP**

The hydroxylamine spin probe 1-hydroxy-3-carboxy-pyrolidine (CP-H) reacts with superoxide radical ($O2^{\cdot-}$) to form the stable 3-carboxy-proxy ($CP^{\cdot}$) radical. Adapted from supplier website (www.axxora.com).

## 2.12 Drug dosing

For some experiments (described in Chapter 6) the beta 1 ($\beta_1$) adrenoreceptor antagonist, metoprolol, was used to investigate the role of the sympathetic nervous system (SNS) in the action of DEP on I/R injury. Metoprolol (dissolved in 0.9% sodium chloride) was given as a single 10 mg/kg ip injection at the time of instillation. Studies in rats have used higher (Beique *et al.*, 2000) or lower doses (Beril Gok *et al.*, 2007); therefore, a dose between these concentrations was selected to ensure an active dose was given, yet minimise non-specific effects. In an additional group, hearts from DEP-instilled animals underwent *ex vivo* I/R while being perfused with Tyrodes buffer containing metoprolol (10 µM final concentration, Kovacs *et al*., 2009), to control for any direct *ex vivo* influence of the drug on injury.

The transient receptor potential (TRP) ion channel super family has been reported to participate in the detection of various sensory stimuli (Eid *et al.*, 2009). Sensory responses following exposure to DEP were evaluated using a TRP vanilloid 1 (TRPV1) receptor antagonist, AMG 9810 ((2E)-N-(2, 3-Dihydro-1, 4-benzodioxin-6-yl)-3-[4-(1, 1-dimethylethyl) phenyl]-2-propenamide, Tocris bioscience, Bristol, UK).

30 mg/kg AMG 9810 was dispersed with the vehicle or DEP-suspension and administered by intra-tracheal instillation (see above; Chapter 2.3). Gavva *et al*. (2005) reported blockade of capsaicin-induced eye wiping after an intraperitoneal injection of 3, 10 and 30 mg/kg AMG 9810. The highest dose was selected in the study because of the relatively short half-life of AMG 9810. The selectivity of action was determined by perfusing the rat hearts with 1 μM AMG 9810 (final concentration).

## 2.13 Drugs and reagents

Unless otherwise stated all pharmacological agents were obtained from Sigma Aldrich (Dorset, UK) and all basic salts were obtained from VWR (Leicestershire, UK). All drugs were dissolved in sterile 0.9% saline, with the exception of CP-H [dissolved in 0.01 M ethylenediaminetetraacetic acid (EDTA; final concentration, 0.1 mM) to minimise metal-ion-induced auto-oxidation].

## 2.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism software (V5.0; GraphPad Software Inc, USA). In each chapter all data are expressed as mean ± SEM. Comparisons were performed by unpaired Student's t test and one-way or two-way analysis of variance (ANOVA) as appropriate. For full details, please see individual chapters. P values less than 0.05 were considered to be statistically significant.

# Chapter 3

# Development of an *in vivo* pre-clinical model for investigating endothelial cell function: drug dosing and route of administration

## 3.1 Introduction

Vascular endothelial cell injury and dysfunction plays an important role in the pathogenesis of many cardiovascular disorders including atherosclerosis, coronary vasoconstriction and myocardial ischaemia (Celermajer, 1997; Endemann *et al.*, 2004; Lerman *et al.*, 2005). In a normal physiological state, healthy endothelium regulates numerous blood vessel functions including the control of vascular tone, coagulation, cell adhesiveness, fibrinolysis, and inflammation (Celermajer, 1997). This is achieved through the synthesis and release of various factors in response to chemical and physical stimuli such as acetylcholine (ACh), bradykinin, thrombin and shear stress (Celermajer, 1997). One of the most important and best characterised substances released from the endothelium is nitric oxide (NO), a potent vasodilator produced through oxidation of L-arginine by endothelial nitric oxide synthase (eNOS) (Furchgott & Zawadski, 1980). Endothelial dysfunction encompasses a range of abnormalities, the most extensively studied being impairment of endothelium-dependent relaxation, mainly as a result of loss of NO bioavailability (Boomsma *et al.*, 1993; Davignon *et al.*, 2004). Under normal conditions, NO produced by endothelium diffuses into vascular smooth muscle where it binds to soluble guanylate cyclase (sGC), increasing cyclic 3', 5' guanosine monophosphate (cGMP) and causing relaxation (Davignon *et al.*, 2004).

In humans, endothelium-dependent vasomotion has been studied in both the coronary and peripheral circulations by measuring changes either in vessel diameter or in regional blood flow in response to vasoactive influences (Wegesser *et al.*, 2008). Forearm venous occlusion plethysmography with local brachial artery infusion has become widely recognised as the 'gold standard' for endothelial cell function testing (Webb & Hand, 1995). First described over 100 years ago by Hewlett and von Zwaluweburg, this approach is less invasive than measurements of coronary endothelial cell function and allows administration of subsystemic doses of

vasoactive factors, thus minimising disturbances to systemic physiology (Hewlett & von Zwaluweburg, 1909).

Previous work from this laboratory has shown both short- and long-term impairment of endothelium-dependent dilation in humans after exposure to diesel exhaust (DE) (Mills *et al.*, 2007; Mills *et al.*, 2005; Tornqvist *et al.*, 2007). These studies are limited, however, in their ability to investigate the mechanisms underlying endothelial cell dysfunction. More recent data from this laboratory have suggested that superoxide radicals contribute significantly to the endothelial cell dysfunction in isolated blood vessels exposed to diesel exhaust particulate (DEP) (Miller *et al.*, 2009). However, this experimental approach assumes that a considerable number of the particles are able to translocate from the lung to the circulation. Whilst studies have demonstrated that such translocation of nanoparticles is feasible (Brown *et al.*, 2002; Kreyling *et al.*, 2002; Oberdorster, 2004) there remains considerable uncertainty over whether this mechanism underlies the health effects of combustion-derived nanoparticles (Brook *et al.*, 2004; Mills *et al.*, 2006).

Given the limitations cited above, there is a need to introduce a model to study the effects of DEP exposure on endothelial cell vasomotor function in the physiological environment under the influence of neuronal, circulatory and local mediators. The work described in this chapter aimed to develop a suitable model for studying endothelial cell vascular function *in vivo*. In general, intravenous administration of vasodilators causes a drop in blood pressure leading to a reflex vasoconstriction which rapidly restores the blood pressure (Dabisch *et al.*, 2008; Takahashi *et al.*, 1996). These vasodilator-induced alterations in blood pressure make changes in blood flow difficult to interpret. This complication can be avoided in studies using decerebrate or pithed animals. An alternative, less technically challenging approach is to inject vasodilators locally into a vascular bed. The clinical studies discussed above assessed endothelial cell function by measuring forearm blood flow responses to endothelium-dependent and –independent vasodilators. In this study we

developed a model to assess rat hind-limb endothelial cell function by measuring femoral artery blood flow (FBF) responses to intra-arterial injections of vasodilators. In support of such a model for endothelial function testing, Dabsich and colleagues successfully demonstrated intra-arterial vasodilator agents to increase rat hind-limb blood flow (HBF) without decreasing systemic blood pressure (Dabisch *et al.*, 2008).

### 3.1.1 Hypothesis

The work described in this chapter addressed the hypothesis that it was possible to study endothelium-dependent and –independent vasodilator responses in the hind-limb vasculature of the rat *in vivo*.

## 3.2 Methods

### 3.2.1 Animals

Adult male Wistar rats (250-350g; Charles River Laboratories) were used in all experiments.

### 3.2.2 Development of a model for studying endothelial cell function *in vivo* in the rat hind-limb

#### 3.2.2.1 Rat hind-limb preparation

All rats were anesthetised with isoflurane (induced at 5%, maintained at 2% in 100% oxygen). The left carotid artery was cannulated and connected to a fluid-filled pressure transducer for continuous monitoring of arterial blood pressures (systolic, diastolic and mean) and heart rate (HR) as described in Chapter 2.7.1.1.

A midline incision was performed in the abdominal wall and the right iliac artery just distal to the aortic bifurcation was carefully isolated from surrounding tissue. Once the artery was isolated, the tip of a Transonic flow probe (type 0.7V; Transonic Systems Inc.) was covered with lubricating jelly (Surgical lubricant sterile bacteriostatic, Fougera) and the probe was placed around the isolated vessel. FBF (millimeters per minute) was monitored continuously throughout the study via a Transonic TS420 flowmeter (Transonic System Inc.). Steady state levels of blood flow were attained before the beginning of the experimental protocol (Figure 3.1).

At the end of the experiment all rats were killed by exsanguination.

### 3.2.2.2 Intravenous administration of endothelium-dependent and –independent vasodilators

The left jugular vein was cannulated in the anaesthetised rat as described in Chapter 2.7.1.1. All studies used ACh as an endothelium-dependent vasodilator and sodium nitroprusside (SNP) to assess endothelium-independent, cGMP-mediated relaxation. Upon attaining a steady blood flow, animals were subjected to single intravnous injections of either ACh (0.4 and 1.8 µg/kg) or SNP (0.30 and 3.0 µg/kg) or an equivalent volume of saline. These doses were based on previous injection experiments in the rat (Dabisch *et al.*, 2004; Dabisch *et al.*, 2008).

### 3.2.2.3 Intra-arterial administration of endothelial-dependent and –independent vasodilators

A left groin incision was made in the anaesthetised rat to gain access to the femoral vessels. The fine connective tissue connecting the left femoral artery, vein and saphenous nerve was carefully cleared by blunt dissection. After isolating the femoral artery, a polyethylene cannula (0.61mm outer diameter (o.d.), 0.28mm inner diameter (i.d.), Portex, Jencons Scientific Ltd, Bedfordshire, UK) was inserted and gently advanced until its tip reached the bifurcation of the abdominal aorta, allowing drug to be administered as it entered the femoral artery of the limb where flow was assessed (Figure 3.1). The cannula was secured in place with three 5-0 braided silk ligatures.

Upon attaining a steady-state blood flow ACh (0.07, 0.7 and 7 µg) and the saline vehicle were delivered in a volume of 10 µl via a microsyringe (25 µl Hamilton) attached to the right femoral artery cannula, followed by increasing doses of SNP (0.09, 0.9 and 9 µg). The doses were calculated to be equivalent to those used for intravenous administration. Each animal received all three doses of ACh, SNP or the saline vehicle administered at random. Blood flow was allowed to return to a new and stable baseline value after administration of test drug or saline vehicle.

### 3.2.3 Statistics

All values are expressed as mean ± SEM. The mean arterial pressure (MAP) was computed as diastolic pressure plus one third of the difference of systolic blood pressure (SBP) minus diastolic blood pressure (DBP). HR was measured as the period of time between successive systolic peaks. Blood flow responses to vasodilators were compared using one-way ANOVA followed by Bonferroni post-hoc test. Changes in haemodynamic parameters in response to different concentrations of vasodilators were analysed by two-way ANOVA using the Bonferroni post-hoc test. $P<0.05$ was considered to be statistically significant.

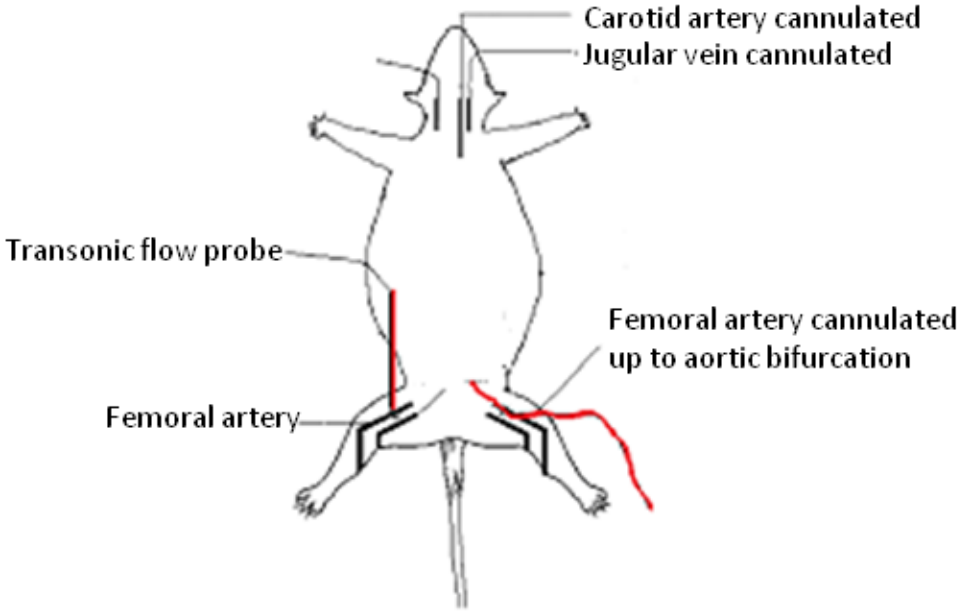


**Figure 3.1 Illustration showing the positioning of cannulae and flow probe.**

Cannulae were inserted into one carotid artery (blood pressure measurements), one jugular vein (acetylcholine (ACh) or sodium nitroprusside (SNP) injections) and one femoral artery (ACh or SNP injections). A transonic flow probe was positioned around the right femoral artery. (Adapted from Mahajan *et al*., 2004).

## 3.3 Results

### 3.3.1 Baseline blood pressure, HR and FBF

The baseline MAP, HR and FBF were 102.4 ± 5.0 mmHg (n=5), 365 ± 10 beats/min (n=5) and 2.6 ± 0.4 ml/min (n=6) respectively.

### 3.3.2 Hind-limb vasodilator responses to intravenous injections of ACh and SNP

The administration of ACh (0.4 µg/kg single iv bolus) did not affect blood flow compared with baseline (Figure 3.2a). Increasing the dose of intravenous ACh to 1.8 µg/kg induced a transient decrease in flow (17% reduction in baseline flow) but this did not reach statistical significance (Figure 3.2a). The response recorded in the hind-limb vascular bed after administration of SNP (0.3-3.0 µg/kg single iv bolus) was similar to that produced by ACh (Figure 3.2b). The two vasodilators caused a temporary significant drop in systemic arterial pressure (Figures 3.3a & b). Intravenous administration of ACh or SNP produced minimal change in HR (Figures 3.3c & d). Saline administered intravenously had no effect on FBF or haemodynamic parameters (data not shown).

### 3.3.3 Hind-limb vasodilator responses to intra-arterial injections of ACh and SNP

The intra-arterial injections of ACh in doses of 0.07 to 7 µg significantly increased FBF over baseline (Figure 3.4a). The administration of 0.07 or 0.7 µg ACh had no significant effect on systemic arterial pressure, whereas 7 µg ACh significantly decreased arterial pressure (Figure 3.5a). Intra-arterial injections of SNP (0.09 and 0.9 µg) into the hind-limb vascular bed significantly increased blood flow over baseline with minimal changes in arterial pressure (Figures 3.4b & 3.5b). Increasing

the dose to 9 µg SNP also significantly increased FBF (Figure 3.4b) but caused a significant drop in arterial pressure (Figure 3.5b). Intra-arterial administration of ACh or SNP produced minimal change in HR (Figures 3.5c & d). The haemodynamic and regional blood flow responses to ACh or SNP were not dose-dependent. Intra-arterial administration of 10 µl saline had no effect on FBF or haemodynamic parameters (data not shown).

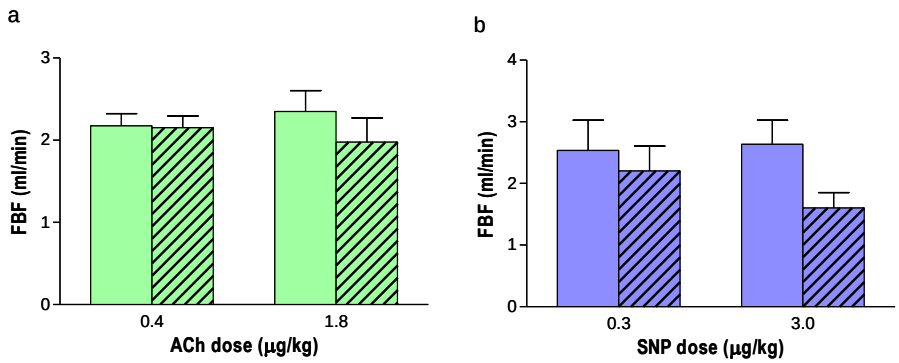


**Figure 3.2 Intravenous administration of acetylcholine (ACh) and sodium nitroprusside (SNP) did not affect femoral artery blood flow (FBF).**

FBF before (filled columns) and after (diagonally striped columns) intravenous injections of (a) ACh (0.4 and 1.8 µg/kg) or (b) SNP (0.3 and 3.0 µg). Columns represent mean ± SEM (n=3-4).

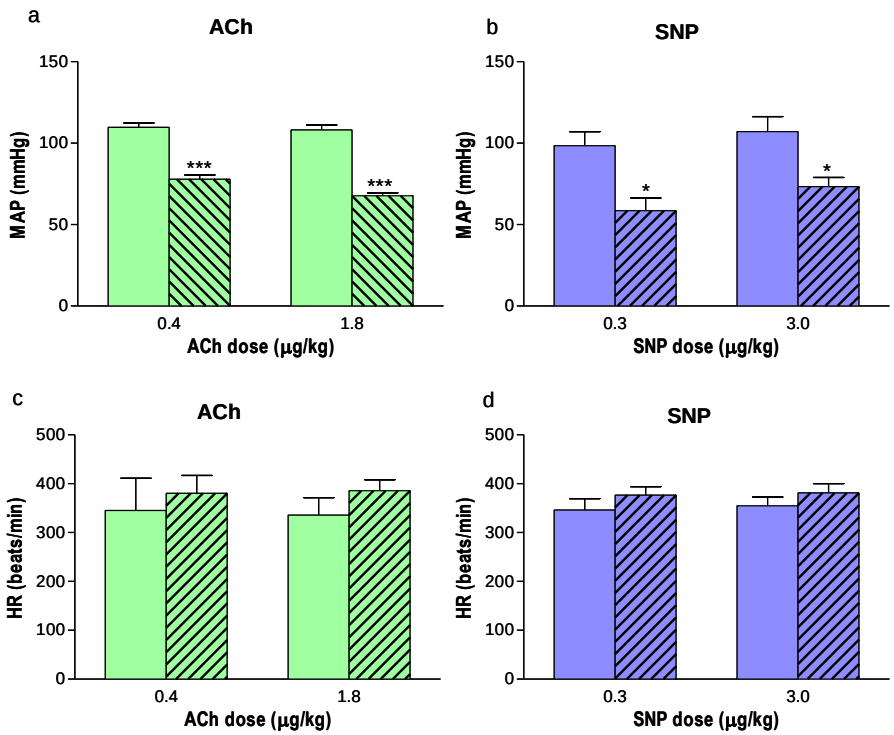


**Figure 3.3 Intravenous administration of acetylcholine (ACh) and sodium nitroprusside (SNP) decreased blood pressure and had no effect on heart rate (HR).**

Mean arterial blood pressure (MAP, a & b) and HR (c & d) before (filled columns) and after (diagonally striped columns) intavenous injections of ACh (0.4 and 1.8 µg/kg) or SNP (0.3 and 3.0 µg). Columns represent mean ± SEM (n=3-4). *P<0.05; ***P<0.001, compared to pre-injection value, two-way ANOVA using the Bonferroni post-hoc test.

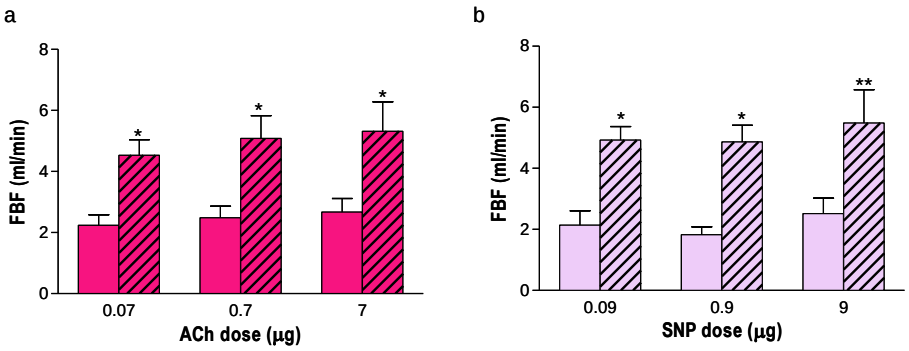


**Figure 3.4 Femoral artery blood flow (FBF) increased in response to intra-arterial administration of acetylcholine (ACh) and sodium nitroprusside (SNP).**

FBF before (filled columns) and after (diagonally striped columns) intravenous injections of (a) ACh (0.07, 0.7 and 7 µg) or (b) SNP (0.09, 0.9 and 9 µg). Columns represent mean ± SEM (n=6). *P<0.05; **P<0.01, compared to pre-injection value, two-way ANOVA using the Bonferroni post-hoc test.

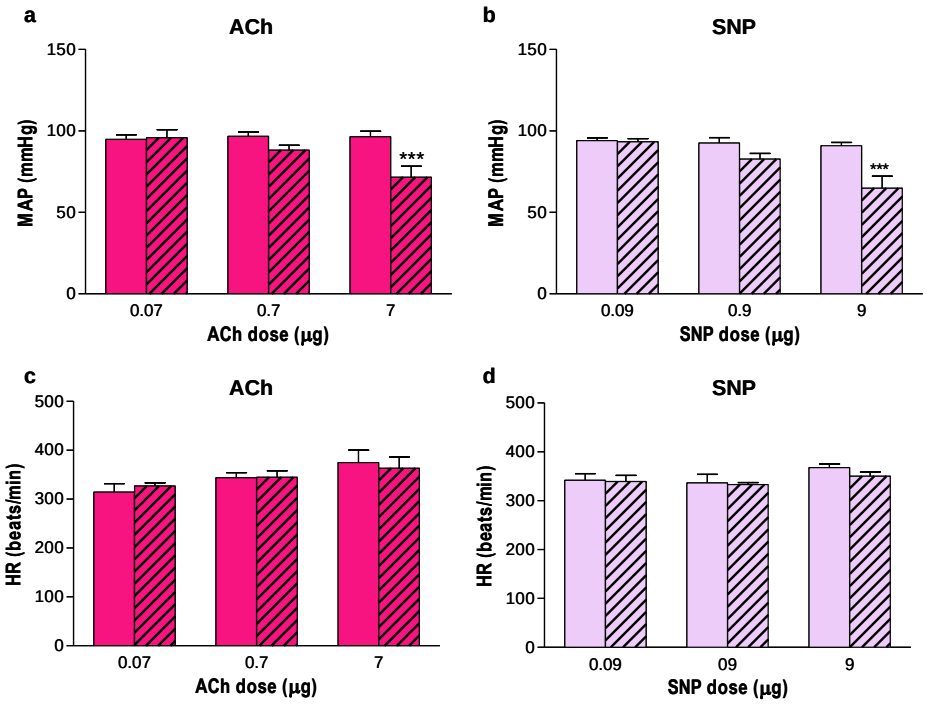


**Figure 3.5 Acetylcholine (ACh) and sodium nitroprusside (SNP) administered intra-arterially did not alter systemic blood pressure, except at the highest dose.**

Mean arterial blood pressure (MAP, a & b) and heart rate (HR, c & d) before (filled columns) and after (diagonally striped columns) intra-arterial injections of ACh (0.07, 0.7 and 7 µg) or SNP (0.09, 0.9 and 9 µg). Columns represent mean ± SEM (n=5). ***P<0.001, compared to pre-injection value, two-way ANOVA using the Bonferroni post-hoc test.

## 3.4 Discussion

The purpose of this study was to develop a model, and assess its reproducibility, for measuring drug-induced changes in blood flow to allow evaluation of endothelial function in the rat hind-limb. This was driven by the need for an *in vivo* approach to study the effects of DEP exposure on endothelial cell vasomotor function. The data presented here confirm that it was possible to administer vasodilators intra-arterially into the regional vascular bed to cause vasodilatation and thus increase blood flow.

In the current study two routes of administration were compared: intravenous and intra-arterial. Single intravenous bolus doses of 0.4-1.8 µg/kg ACh induced a rapid and transient drop in systemic blood pressure. The results of this study showed that intravenously administered SNP (0.3-3 µg/kg) had the same blood-pressure-lowering effect to that of ACh. Analogous results have been reported in rats after intavenous administration of ACh in a dose range of 0.1-3 µg/kg (Dabisch *et al.*, 2008). Intravenous administration of SNP has also been shown to produce dose-dependent decreases in MAP in rats (Takahashi *et al.*, 1996). It is well recognised that baroreceptors in the vessel walls detect the resulting drop in blood pressure causing vasoconstriction which increases blood pressure back to normal range (Dabisch *et al.*, 2008; Saeed *et al.*, 2005; Takahashi *et al.*, 1996). These vasodilator-induced alterations in blood pressure make changes in blood flow difficult to interpret. Thus, it seems likely that reflex vasoconstriction blunted the dilator responses to intravenous ACh and SNP in the current study. Baroreceptor reflex function has been assessed by measuring resultant changes in HR in response to changes in blood pressure induced by vasoactive drugs (Brook *et al.*, 2009a). In the present study, intravenous drug administration had little or no effect on HR. Future studies assessing heart rate variability (HRV) may provide a more sensitive measure for assessing fluctuations in autonomic nervous activities (Kuwahara *et al.*, 1994).

In the present study, experiments were performed to investigate whether vasodilators administered locally into the hind-limb vascular bed may avoid the above-mentioned haemodynamic limitations associated with intravenous administration. The results presented here show, in rats, that intra-arterial injection of the endothelium-dependent vasodilator ACh (0.07 and 0.7 µg) caused an increase in FBF. These results are in agreement with those of previous studies in the rat hind-limb vascular bed (Dabisch *et al.*, 2008). ACh given intra-arterially at doses of 0.0075, 0.075 and 0.75 µg caused significant increases in blood flow in the rat hind-limb (Dabisch *et al.*, 2008). In the present study intra-arterial administration of 7 µg ACh increased blood flow through the femoral artery but a significant decrease in systemic blood pressure was also seen. This is most probably due to ACh entering the systemic circulation. Intra-arterial administration of the endothelium-independent vasodilator SNP (0. 09 and 0.9 µg) was also capable of increasing hind-limb blood flow without altering systemic arterial pressures. This is important for studies evaluating vascular responses in rats following pulmonary exposure to DEP in the next chapter. Using both endothelium-dependent and –independent vasodilators will provide a better knowledge of the cellular mechanisms underlying vascular responses to DEP.

To summarise, this study has shown that it is possible to administer vasodilators into the hind-limb vascular bed to cause local vasodilatation without major effects on the systemic circulation. This protocol was applied in Chapter 4 of this thesis to evaluate endothelial cell function in rats exposed to DEP.

# Chapter 4

## *In vivo* assessment of endothelial cell function after exposure to diesel exhaust particulate (DEP)

## 4.1 Introduction

Previous studies from this laboratory have shown both short- and long-term impairment of endothelium-dependent dilation in humans after exposure to diesel exhaust (DE) (Mills *et al.*, 2007; Mills *et al.*, 2005; Tornqvist *et al.*, 2007). Furthermore, *ex vivo* exposure of blood vessels to DEP inhibits nitric oxide (NO)-mediated vasodilatation via generation of superoxide free radicals (Miller *et al.*, 2009). Thus, DEP can directly alter endothelial cell function but this assumes that a considerable number of the particles are able to translocate from the lung to the circulation. Whilst studies have demonstrated that such translocation of nanoparticles is feasible (Brown *et al.*, 2002; Kreyling *et al.*, 2002; Oberdorster *et al.*, 2004) there remains considerable uncertainty over whether this mechanism underlies the health effects of combustion-derived nanoparticles (Brook *et al.*, 2004; Mills *et al.*, 2006).

An alternative suggestion is that inflammation induced by particulate matter (PM) in the lung develops into a systemic inflammatory response, causing indirect cardiovascular changes (Seaton *et al.*, 1995). Clinical and experimental studies have reported pulmonary inflammation to peak ~ 6-12 hours after exposure to DEP (Madden *et al.*, 2000; Nemmar *et al.*, 2003b; Salvi *et al.*, 1999). Several different types of particulate have been shown to induce pulmonary inflammation (Madden *et al.*, 2000; Nemmar *et al.*, 2003b), but the occurrence and potential role of systemic inflammation following pulmonary exposure to particulates is often inconsistent (Mills *et al.*, 2008; Mills *et al.*, 2005; Swiston *et al.*, 2008). The work described in this chapter was designed to evaluate whether instillation of DEP will cause endothelium dysfunction in rats as a consequence of pulmonary and systemic inflammation. Currently, there are limited studies investigating the potential adverse systemic vascular effects after pulmonary exposure to PM (Cozzi *et al.*, 2006; Hansen *et al.*, 2007; Tamagawa *et al.*, 2008). Moreover, in almost all studies

endothelial cell function was assessed in isolated aortic rings after PM exposure (Cozzi *et al.*, 2006; Hansen *et al.*, 2007; Tamagawa *et al.*, 2008). However, isolated vessels are removed from the influence of circulating neurohormonal factors. In the present study, endothelium-dependent relaxation was assessed *in vivo* in the rat hind-limb vascular bed following intra-tracheal instillation of DEP.

### 4.1.1 Hypothesis

Intra-tracheal instillation of DEP in healthy rats causes pulmonary and systemic inflammation, which in turn leads to vascular endothelial cell dysfunction.

### 4.1.2 Aims

The aims of the work described in this chapter were to determine whether intra-tracheal instillation of DEP:

*i.* causes endothelial vasomotor dysfunction *in vivo*

ii. generates pulmonary inflammation

iii. generates systemic inflammation

iv. alters haemodynamic parameters, including arterial blood pressure and heart rate (HR)

## 4.2 Methods

### 4.2.1 Pulmonary instillation of DEP

Adult male Wistar rats (250-350 g; Charles River Laboratories) were used for all experiments. Rats were intra-tracheally instilled with either DEP (0.5 mg in 0.5 ml saline) or vehicle (saline 0.5 ml). For details of the preparation and instillation process please refer to Chapters 2.2 and 2.3. To ensure that saline instillation itself had no notable effects on the parameters studied, an additional group of rats received no instillation. Animals were sacrificed 6 and 24 hours after instillation by exsanguination.

### 4.2.2 Assessment of rat hind-limb endothelial cell function *in vivo*

Rats underwent anaesthesia and the right carotid artery was catheterised for measurement of arterial blood pressures (systolic, diastolic and mean) and HR as described in Chapter 2.7.1.1. The vasomotor response of the rat hind-limb to intra-arterial injections of endothelium-dependent (acetylcholine, ACh) and –independent (sodium nitroprusside, SNP) vasodilators was studied as described in Chapter 3.2.

### 4.2.3 Assessment of pulmonary inflammation

A bronchoalveolar lavage (BAL) was performed as described in Chapter 2.8. Total and differential cell counts were performed on the BAL fluid. Total protein was detected using a bicinchoninic acid (BCA) kit (Thermo Scientific Pierce). Interleukin-6 (IL-6), tumour necrosis factor alpha (TNFα) and C reactive protein (CRP) levels in BAL fluid were assayed by enzyme-linked immunosorbent assay (ELISA, R & D Systems). For details of all the procedures please refer to Chapter 2.5.

### 4.2.4 A complete blood count and plasma pro-inflammatory cytokine levels

Blood samples were collected after the 6 and 24 hour study period as described in Chapter 2.4. A complete blood count was performed on heparinised blood with the use of a Coulter®$A^c$.T series analyser, as described in Chapter 2.5.4. Circulating levels of IL-6, TNFα and CRP were measured by ELISA (R & D Systems) in plasma generated by centrifugation of whole blood for 5 minutes at 1500 g and 4°C, as described in Chapter 2.5.3.

### 4.2.5 Measurement of plasma catecholamine

A commercially available ELISA (CUSABIO®BIOTECH, USA) was used to measure the total catecholamine (adrenaline and noradrenaline) concentrations in the plasma of decapitated rats as described in Chapter 2.6.

### 4.2.6 Statistics

All values are expressed as mean ± SEM. The mean arterial pressure (MAP) was computed as diastolic blood pressure (DBP) plus one third of the difference of systolic blood pressure (SBP) minus DBP. HR was measured as the period of time between successive systolic peaks. Vasomotor responses were expressed as changes in femoral vascular conductance (FVC). This was done to minimise any alterations in arterial pressure in response to ACh and SNP (discussed in detail in Chapter 3). The FVC was calculated by femoral artery blood flow (FBF) divided by MAP. Haemodynamic and inflammatory indices between the groups were compared by one-way ANOVA followed by Bonferroni post-hoc test. Changes in FVC and haemodynamic parameters in response to different concentrations of vasodilators within a group and between DEP instillation and control groups were compared by two-way ANOVA using the Bonferroni post-hoc test. $P<0.05$ was considered to be statistically significant.

## 4.3 Results

### 4.3.1 Effect of DEP instillation on resting HR and blood pressure

There was no difference in resting HR between the groups (358 ± 8 bpm for non-instilled, 341 ± 22 bpm for 6 hour saline, 373 ± 13 bpm for 6 hour DEP, 337 ± 5 bpm for 24 hour saline and 348 ± 13 bpm for 24 hour DEP (Table 4.1). Resting arterial blood pressure (both systolic and diastolic) remained constant at both time points after saline instillation compared to the non-instilled group. However, in DEP-instilled rats mean resting systolic and diastolic pressures were greater (31% and 37% respectively) than saline-instilled rats 6 hours after DEP (Table 4.1). Blood pressures had almost returned to control values by 24 hours after instillation (Table 4.1). The resting rate pressure product (RPP, HR multiplied by systolic arterial pressure) was calculated to provide an indication of myocardial demand. There was no difference in RPP in rats that received saline instillation compared to non-instilled rats (Table 4.1). However, RPP was significantly higher 6 hours after instillation of DEP ($P<0.05$ compared to 6 hour saline), an effect that was no longer evident at 24 hours post-instillation.

### 4.3.2 Effect of DEP instillation on femoral arterial responses to vasodilator agents *in vivo*

There was no difference in resting blood flow between the groups (2.5 ± 0.4 ml/min for non-instilled, 2.4 ± 0.4 ml/min for 6 hour saline, 2.1 ± 0.3 ml/min for 6 hour DEP, 2.7 ± 0.2 ml/min for 24 hour saline and 2.6 ± 0.3 ml/min for 24 hour DEP). In non-instilled rats, FVC rose by 75 and 125% following intra-arterial injections of 0.07 and 0.7 µg ACh, respectively (Figures 4.1a & b). Responses to ACh were not significantly different in saline, compared to non-instilled or DEP-instilled rats at either 6 hours (Figure 4.1a) or 24 hours (Figure 4.1b) after instillation. Recovery time (time for vascular tone to return to that before ACh injection) was prolonged

and more variable 6 hours after DEP instillation compared to saline control (60.1 ± 24.8 versus 24.5 ± 5.9 seconds after intra-arterial injection of 0.7 µg ACh). However, these changes did not reach statistical significance, perhaps due to the relatively high variation in the DEP group. Recovery time following ACh was not different between saline- and DEP-instilled rats at the 24 hour time point (22.4 ± 3.1 versus 27.4 ± 7.8 seconds after intra-arterial injection of 0.7 µg ACh). Intra-arterial injection of ACh (0.07 and 0.7 µg) produced minimal change in HR or arterial pressure (Table 4.3). Responses were not significantly different in saline- or DEP-instilled rats either 6 hours or 24 hours (Table 4.2).

SNP increased vascular conductance at both doses in the non-instilled animals (Figures 4.1c & d) to an extent that was similar in rats both 6 and 24 hours after saline instillation. 6 hours after instillation of DEP, femoral arterial responses to 0.9 µg SNP were suppressed compared to saline controls (3.0 ± 0.6 versus 0.2 ± 0.5 ml/min, $P<0.01$; Figure 4.1c). A similar result was also obtained at 24 hours after instillation (-0.5 ± 0.8 ml/min, $P<0.05$; Figure 4.1d). Unlike ACh, it was not possible to evaluate the time course of the SNP response in DEP-instilled rats at either time point as these animals failed to restore a stable blood flow. Intra-arterial injection of SNP (0.09 and 0.9 µg) produced minimal change in HR or arterial pressure (Table 4.3). Responses were not significantly different in saline- or DEP-instilled rats at either 6 hours or 24 hours (Table 4.2).

| | Group | | | | |
|---|---|---|---|---|---|
| | Non-instilled | Saline | | DEP | |
| | | 6 h | 24 h | 6 h | 24 h |
| HR (beats/min) | 342 ± 12 | 341 ± 44 | 328 ± 24 | 373 ± 13 | 348 ± 13 |
| SBP (mmHg) | 103.9 ± 5.7 | 105.1 ± 5.2 | 101.3 ± 2.4 | **138.8 ± 13.0**** | 116.9 ± 5.9 |
| DBP (mmHg) | 88.9 ± 5.0 | 98.7 ± 5.0 | 85.3 ± 2.2 | **129.7 ± 13.0**** | 101.8 ± 7.1 |
| RPP (HR X SBP/1000) | 35.0 ± 2.4 | 36.3 ± 5.0 | 33.4 ± 1.3 | **48.9 ± 5.2*** | 39.2 ± 1.6 |

**Table 4.1 Baseline cardiac parameters in non-instilled, saline-instilled and DEP-instilled rats.**

Abbreviations: DEP = diesel exhaust particulate, HR = heart rate, SBP = systolic blood pressure, DBP = diastolic blood pressure, RPP = rate pressure product.

Data are expressed as mean ± SEM (n=4-6). *P<0.05, **P<0.01 compared to saline at same time point; one-way ANOVA with Bonferroni post-hoc test.

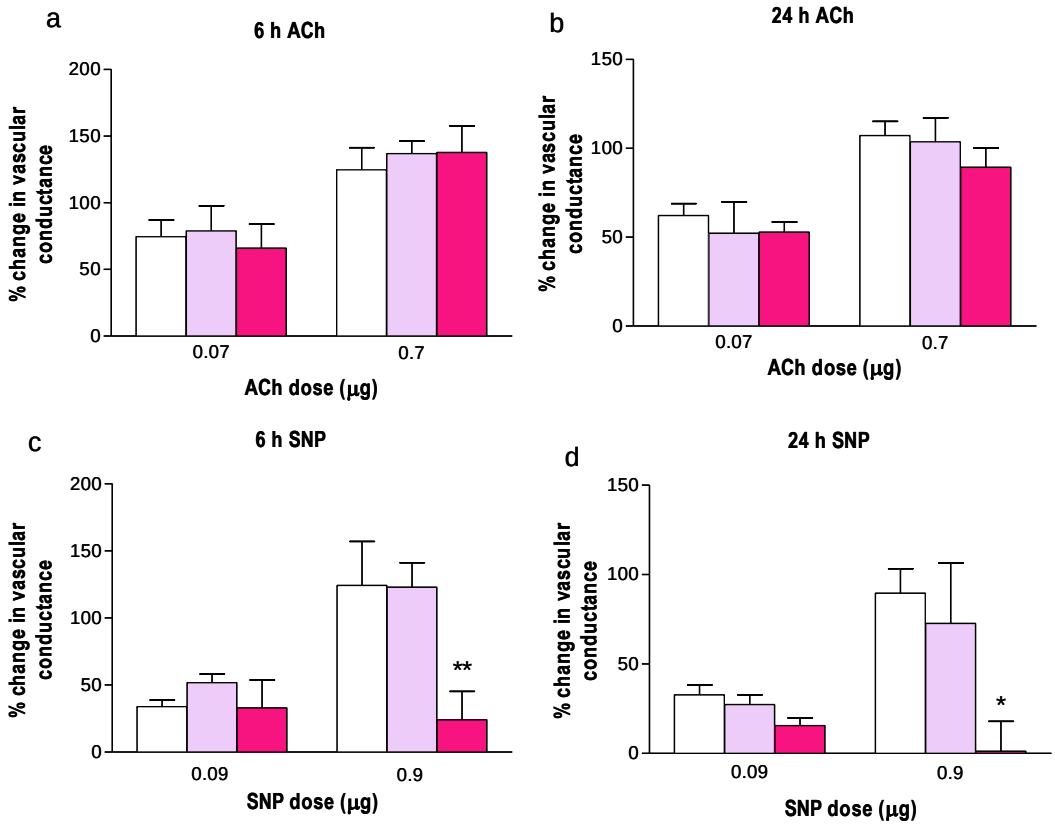


**Figure 4.1 Instillation of diesel exhaust particulate (DEP) does not affect endothelium-dependent relaxation, but impairs vasodilator response to sodium nitroprusside (SNP), *in vivo*.**

Percent change in femoral vascular conductance (FVC) from baseline in response to intra-arterial acetylcholine (ACh; 0.07 & 0.7 µg) and SNP (0.09 & 0.9 µg) 6 hours (a & c) or 24 hours (b & d) after instillation. Responses were obtained in non-instilled rats (open columns), saline-instilled rats (purple columns) or DEP-instilled rats (0.5 mg; pink columns). Columns represent mean ± SEM (n=4-6) *P<0.05, **P<0.01, compared to saline; two-way ANOVA followed by Bonferroni post-hoc test.

| | 0.07 µg ACh | | 0.7 µg ACh | | 0.09 µg SNP | | 0.9 µg SNP | |
|---|---|---|---|---|---|---|---|---|
| | MAP (% baseline) | HR (% baseline) | MAP (% baseline) | HR (% baseline) | MAP (% baseline) | HR (% baseline) | MAP (% baseline) | HR (% baseline) |
| Non-instilled | 96.9 ± 1.0 | 97.6 ± 1.1 | 92.3 ± 2.1 | 97.0 ± 0.1 | 98.3 ± 1.2 | 98.5 ± 1.5 | 90.5 ± 3.4 | 96.5 ± 1.3 |
| **6 h** | | | | | | | | |
| Saline | 93.9 ± 1.2 | 97.7 ± 1.1 | 90.2 ± 4.4 | 97.1 ± 0.1 | 95.9 ± 2.4 | 99.1 ± 2.6 | 88.7 ± 2.0 | 97.4 ± 2.4 |
| DEP | 95.4 ± 1.2 | 97.8 ± 0.7 | 91.8 ± 3.1 | 97.8 ± 0.7 | 94.1 ± 3.1 | 97.1 ± 0.1 | 88.0 ± 1.8 | 97.1 ± 1.0 |
| **24 h** | | | | | | | | |
| Saline | 97.5 ± 1.5 | 102.0 ± 1.5 | 88.4 ± 1.4 | 107.7 ± 4.2 | 99.2 ± 1.1 | 95.3 ± 5.3 | 93.4 ± 2.9 | 100.2 ± 4.9 |
| DEP | 92.7 ± 1.9 | 102.8 ± 3.6 | 91.9 ± 1.4 | 99.1 ± 3.9 | 96.3 ± 0.8 | 98.3 ± 1.6 | 94.7 ± 2.4 | 105.3 ± 4.6 |

**Table 4.2 Effect of vasodilators on cardiac parameters in non-instilled, saline-instilled and DEP-instilled rats.**

Abbreviations: DEP = diesel exhaust particulate, HR = heart rate, MAP = mean arterial blood pressure.

MAP and HR were measured immediately after intra-arterial injection. Data are expressed as mean ± SEM (n=4-7). No significant differences were found between the treatment groups (two-way ANOVA followed by Bonferroni post-hoc test).

### 4.3.3 Effects of DEP instillation on pulmonary cell count and cell differential

Examination of routinely stained cytospins of BAL fluid detected macrophages (large circular cells with a single round nucleus) in all groups (Figures 4.2a-f). DEP particles were found in or adhering to macrophages from rats at both 6 and 24 hours after instillation (Figures 4.2c & e). Large numbers of neutrophils (smaller cells with a multi-lobed nucleus) were visible in the BAL fluid of DEP-instilled rats 6 hours post-exposure (Figure 4.2c), declining markedly by 24 hours (Figure 4.2e). No neutrophils were observed in the lungs of non- or saline-instilled rats.

The values for total and differential cell counts in BAL are shown in Figure 4.3. The total number of cells recovered in BAL fluid from non-instilled rats was $10.3 \pm 2.0 \times 10^5$ cells/ml. At both time points after saline-instillation, there was no difference in the total number of cells compared with those in non-instilled control rats. The total number of cells was increased markedly to $130.5 \pm 35.0 \times 10^5$ cells/ml 6 hours after instillation of DEP ($P<0.001$ compared to saline; Figure 4.3a). By 24 hours total cell counts had decreased to levels that were comparable among all three groups. Differential analysis of BAL cells showed that the BAL from non- or saline-instilled rats was comprised predominately of macrophages (Figure 4.3b). The increase in BAL total cell count 6 hours after instillation of DEP appeared to be attributed to increases in neutrophil numbers; rising from 17.3 % of the total cell count in saline-instilled rats to 75.4% in DEP-instilled rats (Figure 4.3c). At 24 hours after instillation neutrophil numbers had returned to near baseline values.

### 4.3.4 Effect of DEP instillation on total protein level in BAL fluid

Total protein in BAL fluid 6 hours after instillation of DEP was significantly higher than non- or saline-instilled controls ($P<0.001$. Figure 4.4). In contrast, there were no significant differences in protein levels between animals at the 24 hour time point (Figure 4.4).

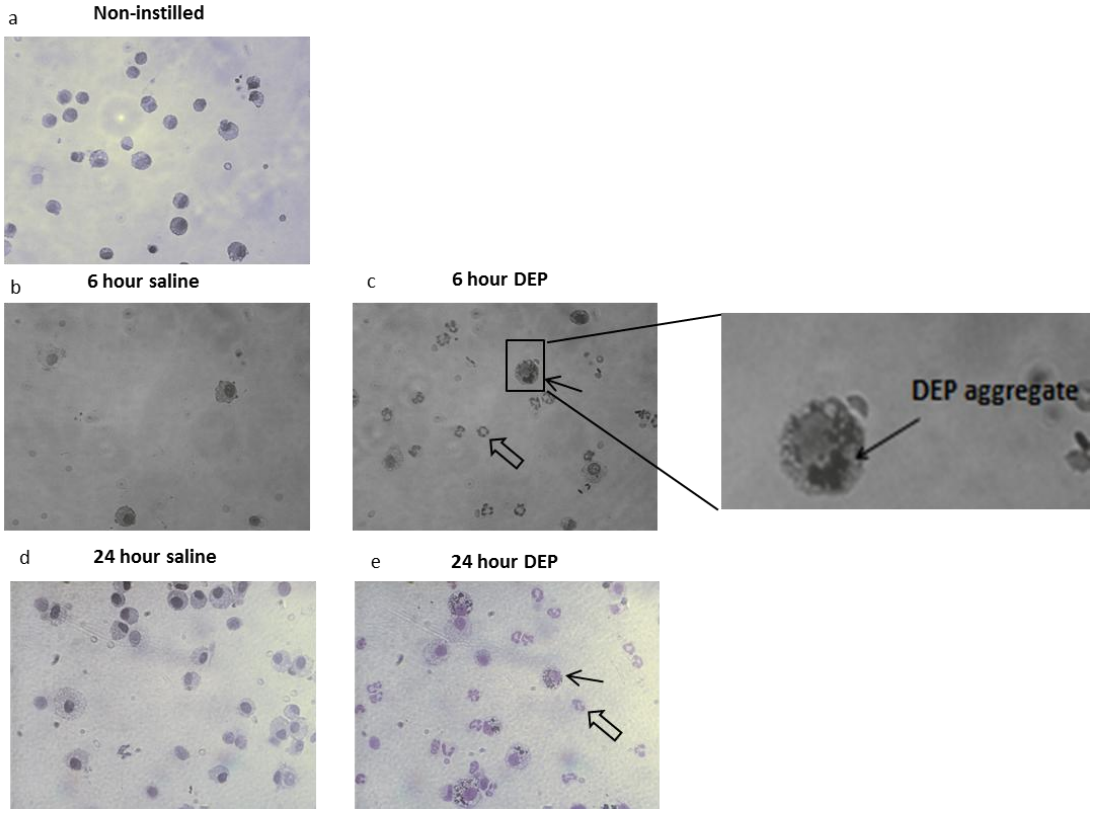


**Figure 4.2 Cytospins of bronchoalveolar lavage (BAL) cells showing pulmonary inflammatory cell infiltration following diesel exhaust particulate (DEP) instillation.**

BAL cells from (a) non-instilled rats, or 6 hours after instillation with (b) 0.5 ml saline or (c) 0.5 mg DEP, and 24 hours after instillation with (d) 0.5 ml saline or (e) 0.5 mg DEP. Open arrows indicate neutrophils; arrowheads indicate alveolar macrophages with aggregates of DEP. Cells stained with Diff-Quik™ physiological stain. Magnification x 40. The outlined area is shown at a higher magnification. All images were acquired at $1x10^5$ cells/slide. Variations in colour were likely a result of slightly different settings on the microscopy computer imaging devise (MCID).

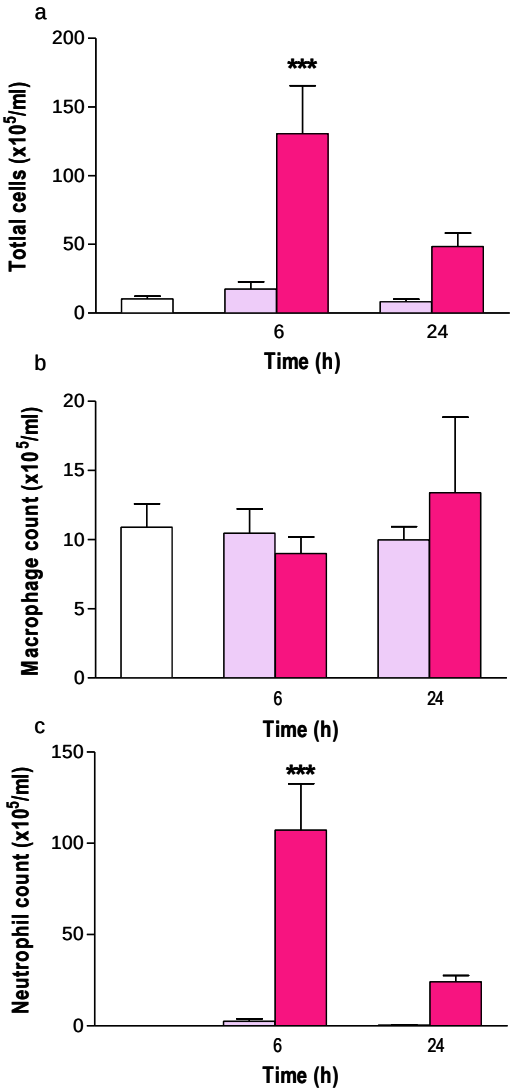


**Figure 4.3 Instillation of diesel exhaust particulate (DEP) causes transient pulmonary inflammation.**

Bronchoalveolar lavage (BAL) fluid was analysed for (a) total cell count, (b) macrophages and (c) neutrophils. Non-instilled (open columns), saline-instilled (purple columns) and DEP-instilled (0.5 mg; pink columns) rats 6 and 24 hours after instillation. Columns represent mean ± SEM (n =5-8). ***P<0.001, compared to saline; one-way ANOVA with Bonferroni post-hoc test.

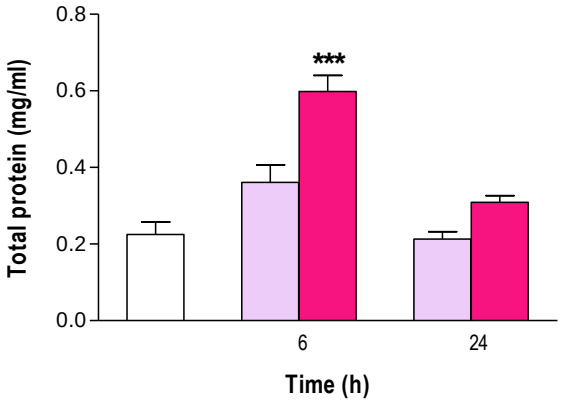


**Figure 4.4 Lung capillary protein leakage at 6 hours after instillation with diesel exhaust particulate (DEP).**

Bronchoalveolar lavage (BAL) fluid was analysed for total protein. Non-instilled (open columns), saline-instilled (purple columns) and DEP-instilled (0.5 mg; pink columns) animals 6 and 24 hours after instillation. Columns represent mean ± SEM (n=6-8) ***P<0.001, compared to saline; one-way ANOVA with Bonferroni post-hoc test.

### 4.3.5 Effect of DEP instillation on pro-inflammatory cytokine levels in BAL fluid

The concentration of IL-6 in BAL fluid from non-instilled rats was 0.30 ± 0.07 ng/ml (Figure 4.5a) and was not altered by saline instillation at any time point. However, the concentration of IL-6 was significantly increased 6 hours after administration of DEP ($P<0.001$compared to the saline; Figure 4.5a). However, by 24 hours the concentration of IL-6 had returned to control levels (Figure 4.5a). The concentration of TNFα levels was below the limit of detection in the non- and saline-instilled rats, but was elevated 6 hours after DEP instillation to 322 ± 190 pg/ml (Figure 4.5b). Within 24 hours, TNFα concentration had fallen close to the limit of detection (50 ± 25 pg/ml; Figure 4.5b). The concentration of CRP was below the limit of detection in the non-instilled rats (Figure 4.5c), but was detectable 6 hours after instillation of either saline or DEP (Figure 4.5c). At 24 hours CRP was undetectable in all groups.

### 4.3.6 Effect of DEP instillation on blood cell count

The circulating white blood cell (WBC) concentration was higher ($8.6 \pm 0.3 \times 10^3/\mu l$) 6 hours after intra-tracheal instillation of DEP compared to saline ($5.8 \pm 0.4 \times 10^3/\mu l$; $P<0.05$; Table 4.3). This effect did not appear to occur at the later 24 hour time point. In contrast, red blood cell (RBC) and platelet counts were unaltered in all treatment groups at both time points (Table 4.3).

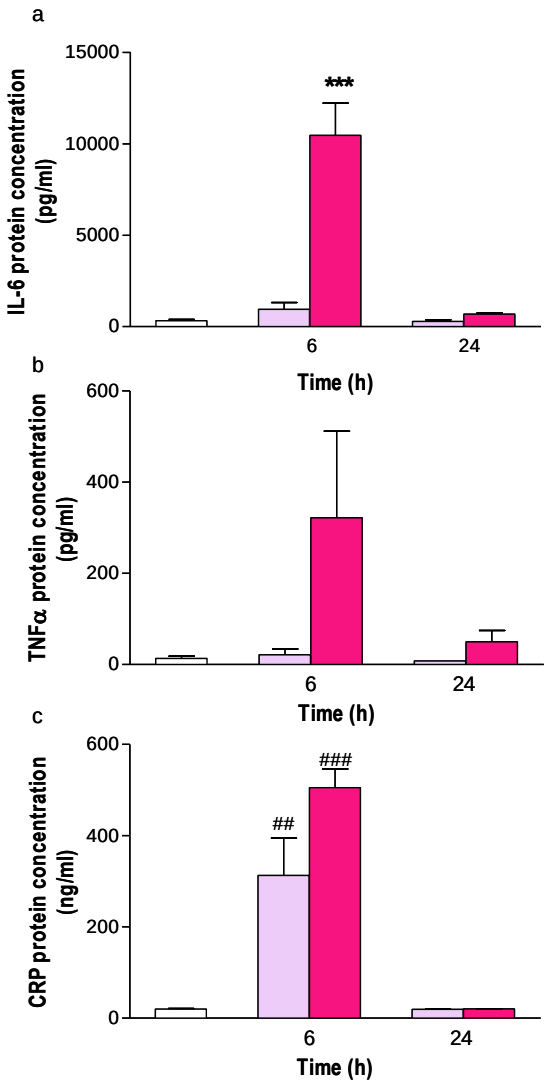


**Figure 4.5 Elevated lung levels of cytokines at 6 hours after instillation with diesel exhaust particulate (DEP).**

(a) Interleukin-6 (IL-6), (b) tumour necrosis factor alpha (TNFα) and (c) C-reactive protein (CRP) in bronchoalveolar lavage (BAL) fluid. Cytokines were detected by enzyme-linked immunosorbent assay (ELISA). Non-instilled (open columns), saline-instilled (purple columns) or DEP-instilled (0.5 mg; pink columns) rats 6 and 24 hours after instillation. Columns represent mean ± SEM (n=4-8) ***P<0.001, compared to saline; ##P<0.01, ##P<0.001 compared to non-instilled; one-way ANOVA with Bonferroni post-hoc test.

| | Group | | | | |
|---|---|---|---|---|---|
| | Non-instilled | Saline | | DEP | |
| | | 6 h | 24 h | 6 h | 24 h |
| WBC ($x10^3$/µl) | 4.6 ± 0.5 | 5.8 ± 0.4 | 4.6 ± 1.0 | **8.6 ± 0.3*** | 5.7 ± 1.4 |
| RBC ($x10^3$/pl) | 5.9 ± 0.3 | 5.4 ± 0.3 | 5.6 ± 0.3 | 5.6 ± 0.2 | 5.3 ± 0.3 |
| Platelets ($x10^3$/µl) | 358 ± 93 | 415± 106 | 525 ± 177 | 447 ± 49 | 618 ± 164 |

**Table 4.3 Effect of DEP instillation on blood cell numbers.**

Abbreviations: DEP = diesel exhaust particulate, WBC = white blood cell, RBP = red blood cell.

Data are expressed as mean ± SEM (n=5). *P<0.05 compared to saline at same time point; one-way ANOVA with Bonferroni post-hoc test.

### 4.3.7 Effect of DEP instillation on blood cytokine levels

Plasma IL-6 concentration in non-instilled rats was 53.5 ± 11.5 ng/ml (Figure 4.6a) and this was not significantly altered 6 or 24 hour after saline instillation (Figure 4.6a). IL-6 concentrations were unchanged 6 hours after DEP instillation but were significantly elevated 24 hours after DEP instillation ($P<0.05$, compared to saline; Figures 4.6a). TNFα concentrations were also increased at 24 hours but not at 6 hours after DEP instillation ($P<0.01$, compared to saline; Figure 4.6b). CRP concentrations were similar between all groups at both time points (Figure 4.6c).

### 4.3.8 Effect of DEP instillation on plasma catecholamine levels

Plasma catecholamine concentrations (adrenaline and noradrenaline) in non-instilled rats were 72.6 ± 0.9 ng/ml and were not altered 6 hours after saline or DEP instillation (Figure 4.7). No data is available at 24 hours after instillation. When carrying out the assay, results of the studies described above had moved the focus towards the earlier 6 hour time point.

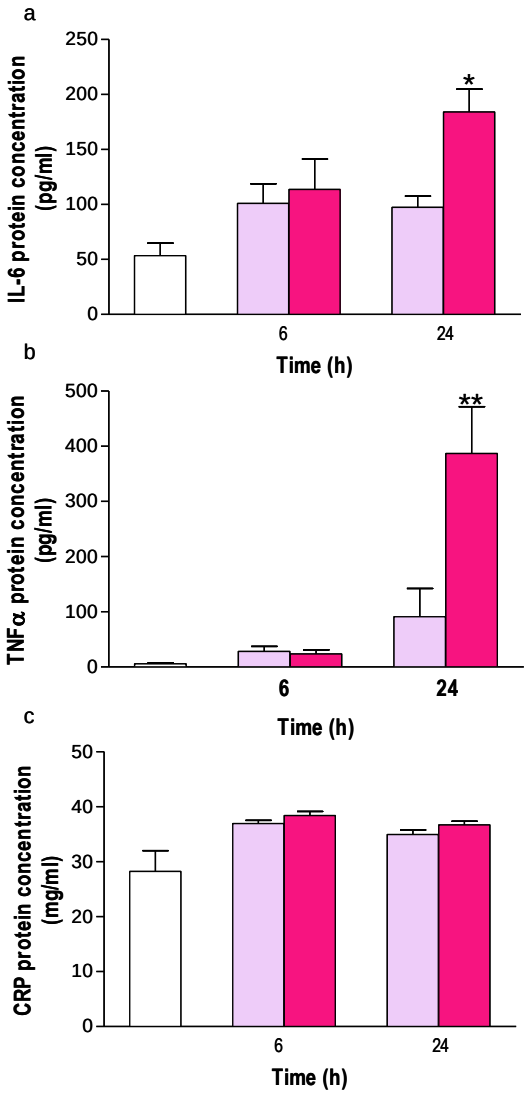


**Figure 4.6 Elevated blood levels of cytokines 24 hours after instillation with diesel exhaust particulate (DEP).**

Plasma (a) Interleukin-6 (IL-6), (b) tumour necrosis factor alpha (TNFα) and (c) C-reactive protein (CRP) concentrations. Cytokines were detected by ELISA. Non-instilled (open columns), saline-instilled (purple columns) or DEP-instilled (0.5 mg; pink columns) rats 6 and 24 hours after instillation. Columns represent mean ± SEM (n=4-6) *P<0.05, **P<0.01, compared to saline; one-way ANOVA with Bonferroni post-hoc test.

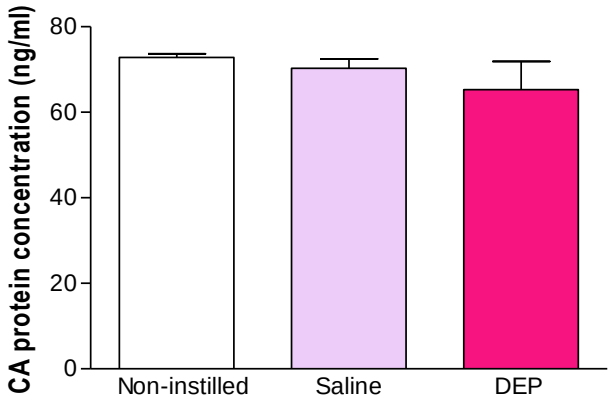


**Figure 4.7 Instillation of diesel exhaust particulate (DEP) does not affect plasma catecholamine concentrations.**

Plasma catecholamine concentrations in non-instilled, saline-instilled and DEP-instilled (0.5 mg) rats 6 hours after instillation. Plasma catecholamine concentrations were detected by enzyme-linked immunosorbent assay (ELISA). Columns represent mean ± SEM (n=3).

## 4.4 Discussion

The purpose of this study was to determine whether pulmonary exposure to DEP impairs or abolishes endothelial cell function secondary to pulmonary and systemic inflammation. Moreover, the knowledge obtained would further our understanding of the role endothelial cell dysfunction plays in cardiac susceptibility to ischaemia/reperfusion (I/R) injury in later chapters of this thesis. DEP exposure induced a pulmonary and systemic inflammation with no effect on endothelium-dependent vasodilation in the rat hind-limb vasculature. Pulmonary inflammation was detectable at 6 hours post-exposure. In contrast, systemic inflammation was detectable at 24 hours post-exposure, by which time the pulmonary inflammation had mostly resolved.

### 4.4.1 Influence of DEP instillation on vascular function in healthy male rats

Previous studies from this laboratory have shown both short- and long-term impairment of endothelium-dependent dilation in humans after exposure to DE (Mills *et al.*, 2007; Mills *et al.*, 2005; Tornqvist *et al.*, 2007). Inhalation exposure of rats to DE has also been associated with endothelial dysfunction *in vitro* (Cherng *et al.*, 2009; Knuckles *et al.*, 2008; Kodavanti *et al.*, 2011). There is some indication that these effects are mediated by the particulate components of the DE (Lucking *et al.*, 2011; Mills *et al.*, 2011).

Particles such as those generated from DE engines and ambient air have been shown to attenuate vascular function in various rodent models (Cozzi *et al.*, 2006; Hansen *et al.*, 2007; Kido *et al.*, 2011; LeBlanc *et al.*, 2009; Nurkiewicz *et al.*, 2008; Tamagawa *et al.*, 2008), although vascular impairment is not always observed despite the exposure to high doses of particulate (Bagate *et al.*, 2004b; Gerlofs-Nijland *et al.*, 2010). The reason for these discrepancies is likely to be multi-factorial, being influenced by type of particle, dose and duration employed, animal species, the type of vessel examined and where/when the measurements were made.

It is also likely that negative findings may by under-represented due to the tendency not to report negative data. The experiments presented in this chapter were specifically designed to maximise the chances of detecting alterations in vascular function by making measurements *in vivo*, in the presence of neurohormonal factors. In the anaesthetised rat, both the endothelium-dependent vasodilator ACh and the endothelium-independent vasodilator SNP induced an increase in blood flow to the hind-limb. Both agents were delivered locally via the femoral artery to avoid reduction of systemic blood pressure and the associated reflex vasoconstriction (Dabisch *et al.*, 2008; Takahashi *et al.*, 1996). In rats that had received DEP 6 hours prior to assessment of vascular function, responses to ACh in the hind-limb resistance bed were not different from those achieved in saline-instilled rats. This contrasts with earlier clinical studies in the laboratory demonstrating a decrease in the vascular resistance in the human forearm after DE exposure (Mills *et al.*, 2008; Mills *et al.*, 2007; Mills *et al.*, 2005). It is, however, difficult to make direct comparisons between the *in vivo* pre-clinical and clinical studies. The clinical studies used whole DE, comprising both a particle-phase and gas-phase. Additionally, the route of exposure varied between these studies (inhalation versus instillation). Parallel studies in my own group explored the relaxation of femoral arteries *ex vivo* in response to ACh in DEP-instilled rats (see Appendix 2). These studies found no underlying alteration in the endothelium-dependent vasodilator function of isolated arteries in saline- or DEP-instilled rats, supporting the *in vivo* measurements (see Appendix 2). Similarly, DEP-instillation had no effect on ACh-induced relaxation in the aorta (a large conduit artery) or in mesenteric resistance arteries *ex vivo* (see Appendix 2). Using similar concentrations of DEP to those reported in this study, Hansen *et al*. (2007) reported intra-peritoneal injection of DEP to have no effect on endothelial function in rat aortic rings of healthy rats *ex vivo*. The passage of intra-tracheally instilled DEP from the lung to the systemic circulation cannot be ruled out in the current study. However, it is clear that any potentially translocated particles are not altering endothelial cell function.

Endothelium-independent relaxation in response to SNP *ex vivo* was also unaffected by DEP instillation, suggesting that sensitivity of the smooth muscle to SNP was not

compromised (see Appendix 2). In contrast, the increase in FBF induced by SNP *in vivo* was impaired 6 and 24 hours after administration of DEP. The reason for these discrepancies between the *in vivo* and *ex vivo* data are unknown and require further study. With no effect of DEP instillation on SNP-related vascular responses in the isolated vessels, it would suggest involvement of a neurohormonal activation rather than altered cellular function within the arterial wall. In the current study, arterial pressures (discussed in detail below) were elevated 6 hours after instillation of DEP, suggestive of increased sympathetic activity. These data provide further support to the hypothesis that the impairment in the SNP-induced vascular response was due to a neurohormonal change. Furthermore, this is also consistent with studies in animals (McQueen *et al.*, 2007; Rhoden *et al.*, 2005) and in man (Gold *et al.*, 1999; Pope *et al.*, 1999) reporting associations between baroreflex function, autonomic nervous system (ANS) activity and particulate air pollution. The mechanism of these effects, such as the involvement of changes to sympathetic activity and baroreflex sensitivity is yet to be determined, although the role of the sympathetic nervous system (SNS) is explored in relation to myocardial infarction in Chapter 6.

In summary, instillation of DEP does not impair endothelial cell function in the hind-limb vascular bed. Further studies are needed to determine whether the results in the hind-limb vascular bed extend to other vascular beds, including the coronary vascular bed.

### 4.4.2 Pulmonary effects of DEP instillation in healthy male rats

Inflammation has been implicated in many of the actions of DEP and other particles, including actions on the cardiovascular system (Seaton *et al.*, 1995). Much research has been, and is being, undertaken to characterise the exposure of pollutants on lung inflammation. In the present study, lung inflammation was assessed by BAL 6 and 24 hours after particulate exposure to validate the use of the method for delivering DEP to the lungs. DEP induced a neutrophilic airway inflammation 6 hours after exposure, which had largely resolved by 24 hours. Analogous results have been reported in rats 6 hours after intra-tracheal instillation of ultrafine carbon black (CB) particles (Li *et al.*, 1997). Airway neutrophilic inflammation has also been detected

at this time in healthy human volunteers exposed to DEP (Nordenhall *et al.*, 2000; Salvi *et al.*, 1999). In contrast, levels of neutrophils in BAL fluid recovered from rats were significantly elevated 12 hours after intra-tracheal instillation of 1 mg/kg DEP and remained elevated for up to 24 hours (Yokota *et al.*, 2008b). Exposure to concentrated ambient PM has also been reported to induce pulmonary inflammation in mice 24 hours after intra-tracheal instillation of ambient PM (Wegesser *et al.*, 2008). These results suggest that the kinetics of the inflammatory response may be dependent on animal species and nature of particles.

In the present study, the transient lung inflammatory response to DEP may result from direct effects of DEP on airway cells. Increased cytokine production has been reported in alveolar and bronchial epithelial cell lines after exposure to DEP and other air pollution particles (Boland *et al.*, 1999; Boland *et al.*, 2000; van Eeden *et al.*, 2001). Becher *et al*. (1996) reported release of IL-6 and TNFα by rat alveolar macrophages stimulated with PM.

Rats instilled with 0.5 ml of saline showed no significant changes in the number of inflammatory cells in BAL fluid at 6 and 24 hours post instillation compared with the non-instilled control rats. Similar findings were reported in a previous study, where no differences were detected between saline-instilled and non-instilled rats in cellular profiles in BAL fluid (Tesfaigzi *et al.*, 2000). Somewhat unexpectedly, BAL levels of CRP were significantly elevated above controls in both saline-instilled and DEP-instilled animals at 6 hours. This may be due to contamination of the saline. However, this seems unlikely given that sterile saline was used and no effects were noted for the other inflammatory mediators measured.

To summarise, these findings suggest that the lack of effect of DEP exposure on endothelial cell function was not due to ineffective DEP exposure.

### 4.4.3 Systemic effects of DEP instillation in healthy male rats

It has been proposed that the adverse cardiovascular effects of air pollutants are due to, or exacerbated by, the release of inflammatory mediators from the lung into the

circulation. Studies both in humans and in animals have shown air particles to increase the number of polymorphonuclear leukocytes (PMN) in the circulation by inducing rapid release from the bone marrow into the peripheral circulation (Ghio *et al.*, 2011; Mukae *et al.*, 2001; Suwa *et al.*, 2002; Tan *et al.*, 2000). As described above, increased levels of IL-6 were detected in BAL 6 hours after intra-tracheal instillation of DEP. IL-6 released from alveolar macrophages has been previously shown to enhance release of PMNs from the bone marrow (Suwa *et al.*, 2002). In the present study, circulating leukocytes were increased 6 hours, but not 24 hours after intra-tracheal instillation of DEP. These findings are consistent with reports of higher circulating leukocyte counts 4 hours, but not 12 hours, following intra-tracheal instillation of DEP in rats (Yokota *et al.*, 2008b). In contrast, to the results from the WBC counts in the present study, instillation of DEP did not significantly affect RBC count. Other studies in the literature have been inconsistent with respect to RBC counts, reporting increases (Rivero *et al.*, 2005), decreases (Seaton *et al.*, 1999) and no change (Nemmar *et al.*, 2007; Ogugbuaja *et al.*, 2001). Exposure to concentrated air pollution has been reported to lower the anti-oxidative enzyme activities of RBCs in elderly patients (Delfino *et al.*, 2008). The RBCs play an important role in protecting cells against oxidative stress (Tsantes *et al.*, 2006). Changes in the anti-oxidative capacity of the RBCs in response to DEP exposure cannot be ruled out in the present study and warrants consideration in future studies.

Whilst the pulmonary actions of DEP are relatively consistent in the literature, measures of particle-induced systemic inflammatory responses are notoriously variable (Gottipolu *et al.*, 2009; Nemmar *et al.*, 2011; Yokota *et al.*, 2008b). In this study, concentrations of the studied inflammatory mediators (IL-6, TNFα and CRP) were not elevated at 6 hours after DEP instillation, a time at which lung inflammation was detected. Plasma IL-6 and TNFα concentrations were, however, markedly increased 24 hours after DEP instillation compared with the saline control. Similarly, IL-6 levels were highly elevated in mouse plasma 24 hours after intra-tracheal instillation of DEP. IL-6 and TNFα have also been shown to be elevated in the serum and plasma of humans 24 hours, but not 6 hours, after exposure to DE (Mills *et al.*, 2005; Tornqvist *et al.*, 2007). Later systemic changes when the

pulmonary effects induced by DEP are largely resolved has been reported in mice exposed to coal fly ash (CFA) (Smith *et al.*, 2006). These investigators reported systemic inflammation to peak after pulmonary inflammation had already subsided. The time-course of these responses could suggest that these factors originate from the prior inflammatory response in the lung. A recent study in mice showed that inflammatory mediators such as IL-6 translocate from the lung following PM exposure into the blood stream contributing to the systemic inflammation (Kido *et al.*, 2011). Previous studies in animals (Upadhyay *et al.*, 2010; Zhao *et al.*, 2010) and man (Hertel *et al.*, 2010; Peters *et al.*, 2001b; Sandhu *et al.*, 2005) have reported raised levels of CRP in the blood following exposure to urban particulates or episodes of high air pollution, yet results presented here did not demonstrate any changes in this mediator after air pollution. Differences in the type and composition of the particulate or co-pollutant may explain these discrepancies. It is important to note that the results of this study cannot rule out the possibility of that other biomarkers are more sensitive at detecting systemic inflammation.

As noted above, this study detected elevated WBC counts and increased BAL TNFα concentrations 6 hours after instillation of DEP. It is reported that TNFα can induce the expression of adhesion molecules on PMN and endothelial cells (Gamble *et al.*, 1985; Lee *et al.*, 2006; Takahashi *et al.*, 1996). Adhesion molecules play an important role in PMN attachment and subsequent transmigration through vascular endothelium (Walzog *et al.*, 2000). PMN adhesion and transmigration play an important role in many inflammatory disorders, including reperfusion injury (Jordan *et al.*, 1999; Saeed *et al.*, 2005). PMN adhesion molecule expression and function in response to DEP instillation requires investigation, and will be discussed in the next chapter. DEP has been reported to induce interleukin-8 (IL-8) in cultured normal human bronchial epithelial cells (Bayram *et al.*, 1998). This inflammatory cytokine has been identified as an important PMN chemotactic factor (Standiford *et al.*, 1990) and will also be discussed in the next chapter.

To summarise, markers of systemic inflammation could be detected after 24 hours. The time course of these responses could suggest that these inflammatory factors originate from the prior inflammatory response in the lung.

### 4.4.4 Haemodynamic effects of DEP instillation in healthy male rats

During ischaemia there is an imbalance between the heart's oxygen supply and demand. RPP is a major determinant of oxygen consumption. RPP, calculated as the product of SBP multiplied by HR, has been reported to be a valid and reliable indicator of myocardial oxygen consumption (Nelson *et al.*, 1974). Not all changes in myocardial oxygen consumption are determined by HR x SBP. Other major determinants of myocardial oxygen consumption are ventricular wall stress (afterload) and contractility (inotropy), although it was not practical to measure these parameters in the present study. At baseline, RPP was higher 6 hours, but not 24 hours, after instillation with DEP relative to their respective saline-instilled controls. This was principally due to elevated systolic pressures with little or no change in HR. However, it is important to emphasis that calculating HR from the consecutive systolic peaks on the blood pressure tracing may not have been the most accurate approach to detect changes in HR. A better way may have been to calculate HR from an ECG. Regardless, the RPP responses to DEP in rats after intra-tracheal instillation lends additional support to the results of other studies showing a positive association between PM and RPP (Bartoli *et al.*, 2009b; Shah *et al.*, 2008; Urch *et al.*, 2005). However, this study did not examine the impact of DEP instillation on the supply of myocardial oxygen. It would be valuable to investigate the effects of DEP instillation on the RPP when myocardial oxygen supply is interrupted in ischaemia. This will be further investigated in the next chapter.

Diastolic pressures were also higher after DEP instillation. Epidemiological studies and human and animal controlled exposure studies on the associations between air PM and blood pressure have been somewhat inconsistent (Brook *et al.*, 2009a). A study by Dvonch *et al*. (2009) in the Detroit metropolitan area reported a 3.2 mmHg increase in SBP for every 10 $\mu g/m^3$ rise in PM. A chamber exposure study conducted by our laboratory reported no change in blood pressure 6 hours following

exposure to DE compared to filtered air (Mills *et al.*, 2005). Similar haemodynamic results were obtained in controlled human exposure studies using concentrated ambient particles (CAP) (Mills *et al.*, 2008). Controlled animal exposure studies have reported similar discordant results. This may be partly explained by differences in the methodologies of exposure. Vincent *et al*. (2001) reported an increase in blood pressure 2-48 hours after nose-only inhalation exposure to diesel soot in rats. On the other hand, intravenous injections of DEP at doses of 0.02 – 0.5 mg/kg decreased blood pressure in rats 24 hours after dosing (Nemmar *et al.*, 2007). Inconsistencies may also result from using different blood pressure measurement techniques and different PM characteristics and source type. It is possible that the increased plasma IL-6 concentrations 24 hours after DEP instillation may have prevented increases in arterial pressure. Indeed, Tateishi *et al*. (2007) reported in septic patients, an association between high circulating IL-6 concentrations and a decline in blood pressure.

Several plausible mechanisms may explain this increase in blood pressure 6 hours after DEP instillation. Alterations in endothelial function and subsequent changes in vasomotor tone may explain the pro-hypertensive response observed 6 hours after intra-tracheal instillation of DEP. Sun *et al*. (2005) reported PM-induced vasoconstriction in mice to be mediated by systemic inflammatory and oxidative stress responses. In the present study the blood pressure change 6 hours post exposure were not accompanied by changes in systemic cytokine levels. It is, therefore, unlikely that the pro-hypertensive response was due to inflammation-induced vascular dysfunction. In further support of this conclusion, as discussed above there was no loss of endothelium-mediated vasodilator responses in the hind-limb after DEP instillation. Previous studies both in humans and in animals have reported endothelin-1 (ET-1)-induced haemodynamic changes following exposure to air pollutants (Bouthillier *et al.*, 1998; Calderon-Garciduenas *et al.*, 2007; Peretz *et al.*, 2008b; Vincent *et al.*, 2001). Circulating plasma levels of ET-1, a potent vasoconstrictor peptide released by vascular endothelial cells, were not measured in the present study. It seems more likely that the rapid and transient hypertensive response is mediated by an imbalance of the ANS favouring an increase in the

sympathetic drive. This hypothesis is supported by animal and human studies showing PM-induced elevations in blood pressure to be mediated by neurohumoral activation (Bartoli *et al.*, 2009b; Brook *et al.*, 2009a). Bartoli *et al*. (2008) demonstrated that the raised DBP during a 5 hour exposure to CAP was significantly attenuated by prazosin, a selective alpha 1 ($\alpha_1$) adrenoceptor antagonist. Furthermore, increased sympathetic activation has been suggested from studies reporting associations between air pollutants and decreased heart rate variability (HRV) (Brook *et al.*, 2009a; Gold *et al.*, 1999; Pope *et al.*, 1999; Rivero *et al.*, 2005; Schulz *et al.*, 2005). Interestingly, a recent observational study reported wearing a facemask to significantly lower SBP in healthy subjects during a 2 hour walk in Beijing (Langrish *et al.*, 2009b). The magnitude of the SBP decrease was strongly associated with increases in HRV, reflecting an autonomic regulation of blood pressure (Langrish *et al.*, 2009b). In the present study, there was no difference in plasma catecholamine levels between the groups. However, there are many practical concerns (Boomsma *et al.*, 1993; Goldstein, 2003) about the measurement of catecholamines (such as stability) making it difficult to draw any firm conclusions. A better alternative to measuring sympathetic nerve activity would have been to use microneurography (Nakamura *et al.*, 2003). Unfortunately, this was not available in the laboratory. Importantly, PM exposure studies have also reported increases in parasympathetic tone. Indeed, HRV has been shown to increase in response to particulate air pollution (Brito *et al.*, 2010; Riediker *et al.*, 2004). Together, these studies suggest that air pollution can affect both the sympathetic and parasympathetic branches of the ANS. In rats, Rhoden *et al*. (2005) demonstrated intra-tracheal instillation of CAP to increase both sympathetic and parasympathetic stimulation. A similar observation has been seen in dogs exposed to CAP (Godleski *et al.*, 2000). Enhancement of both sympathetic and parasympathetic tone could explain the lack of change in HR observed in this study.

In summary, the rapid-onset of the DEP-induced elevation in blood pressure is likely to be principally mediated by rapid changes in ANS balance favouring SNS-mediated arterial vasoconstriction.

### 4.4.5 Conclusions

The results obtained clearly show that, instillation of DEP in rats causes both pulmonary and systemic inflammation, but this is not associated with impaired endothelial cell function at 6 and 24 hour post exposure. However, DEP administration produced specific impairments in vascular response *in vivo*, through a mechanism that may involve changes in autonomic activity. The DEP-induced pulmonary inflammation was observed at 6 hours post exposure and largely resolved by 24 hours. Arterial blood pressure and myocardial work and oxygen consumption were also higher 6 hours after exposure to DEP, with less pronounced effects at the later time point. While there was no indication of systemic inflammation at 6 hours after DEP instillation, the levels of two inflammatory mediators, IL-6 and TNFα were increased in the plasma by 24 hours after exposure. The time course of these responses could suggest that these factors originate from the prior inflammatory response in the lung. The following chapter explores the effect of DEP on the susceptibility of rats to ischaemia and reperfusion, using a surgical model to mimic the effects of coronary occlusion in cardiovascular disease. All remaining studies will be carried out at 6 hours post-exposure since the results presented in this study found pulmonary and haemodynamic changes associated with DEP exposure to be more pronounced here than at the later 24 hour time point.

# Chapter 5

# Effect of diesel exhaust particulate (DEP) instillation on cardiac susceptibility to ischaemia/reperfusion (I/R) injury

## 5.1 Introduction

There is growing clinical and experimental evidence for detrimental effects of diesel exhaust (DE) and particulate matter (PM) on coronary vascular function, on atherosclerotic plaque development and stability and on thrombosis and clot resolution (Kunzli *et al.*, 2005; Nemmar *et al.*, 2003a; Suwa *et al.*, 2002). While the coronary effects of PM increase the likelihood of heart attack occurrence there is also evidence that exposure to PM has deleterious effects on the myocardium itself. Particulates and other air pollutants increase the incidence of ventricular arrhythmias in patients with implantable defibrillators (Dockery *et al.*, 2005; Peters *et al.*, 2000; Vedal *et al.*, 2004). Experimental studies have also reported cardiac abnormalities, including impaired left ventricular diastolic performance and arrhythmias after *in vivo* exposure to PM (Anselme *et al.*, 2007; Okayama *et al.*, 2006; Wold *et al.*, 2006; Yan *et al.*, 2008). These observations suggest that exposure to PM may also render the heart more vulnerable to injury following infarction. To date, only one study has addressed this question directly. Myocardial I/R injury was increased in mice 24 hours after pulmonary instillation of concentrated ambient particles (CAP) (Cozzi *et al.*, 2006). However, the mechanism by which pulmonary exposure to pollution is associated with enhanced reperfusion injury is currently unclear.

This chapter assessed the myocardial susceptibility to I/R injury, both *in vivo* and *ex vivo*, in rats that had received a single intra-tracheal exposure of DEP 6 hours earlier. *In vivo* and *ex vivo* animal models of myocardial I/R injury have been extensively used in experimental investigations (Bagate *et al.*, 2006b; Cozzi *et al.*, 2006; Jeanes *et al.*, 2008; Wold *et al.*, 2006). In both models of myocardial I/R injury, ischaemia is induced by transient ligation of the left anterior descending (LAD) coronary artery followed by reperfusion. In these experimental models, it has been shown that initiating reperfusion after ischaemia lasting longer than 20 minutes leads to irreversible myocardial damage (Park *et al.*, 1999). The current study used a model of I/R rather than one of permanent ligation to mimic more closely the clinical setting of reperfusion therapy in acute myocardial infarction (MI). Indeed, these animal models produce changes in cardiac rhythm and contractility similar to those

seen in the clinic (Park *et al.*, 1999; Yellon *et al.*, 2007). The inflammatory infiltrate that surrounds infarcted myocardium also closely resembles the human situation (Park *et al.*, 1999; Yellon *et al.*, 2007). Using both *in vivo* and *ex vivo* models will enable a more accurate and thorough exploration of the molecular mechanisms underlying the effects of PM exposure on I/R injury, extending the previous findings in animal studies.

### 5.1.1 Hypothesis

Intra-tracheal instillation of DEP in healthy rats will increase the vulnerability of the heart to subsequent myocardial I/R injury.

### 5.1.2 Aims

The aims of the work described in this chapter were to determine whether intra-tracheal instillation of DEP:

v. increases susceptibility to *in vivo* I/R injury
vi. increases susceptibility in *ex vivo* I/R injury
vii. increases cardiac oxidative stress
viii. influences myocardial tissue injury and may enhance circulating interleukin-8 (IL-8) levels and neutrophil activation prior to I/R

## 5.2 Methods

### 5.2.1 Pulmonary instillation of DEP

Adult male Wistar rats (250-350 g; Charles River Laboratories) were used for all experiments. Animals were instilled intra-tracheally with either DEP (0.5 mg in 0.5 ml saline) or vehicle (saline 0.5 ml). For details of the preparation and instillation process please refer to Chapters 2.2 and 2.3. To ensure that saline instillation itself had no notable effects on the parameters studied, an additional group of rats received no instillation. Animals were sacrificed 6 hours after instillation by exsanguination. This time point was chosen based on results described in Chapter 4.

### 5.2.2 Coronary artery ligation

Ischaemia induced by coronary artery ligation followed by reperfusion was carried out on rat hearts *in vivo* and *ex vivo*. For details of the two animals models of myocardial I/R injury please refer to Chapter 2.7.

### 5.2.3 Detection of reactive oxygen species (ROS) by electron paramagnetic resonance (EPR) spectroscopy

In some *ex vivo* perfused hearts, oxygen free radical generation from the myocardium was assessed in perfusion fluid using spin label EPR. Measurements were taken at baseline, after 0 and 30 minutes of ischaemia and after 0, 1 and 2 hours of reperfusion. Please refer to Chapter 2.11 for more details.

### 5.2.4 Tissue collection

For histological analysis hearts were cut longitudinally from apex to base. This was followed by fixation, processing and embedding in paraffin wax as described in Chapter 2.8. For infarct size assessment the whole heart was excised and washed in saline to remove excess Evans Blue prior to storage at -20°C. Wet weight was

recorded in some *ex vivo* perfused hearts following reperfusion prior to storage at -20°C.

### 5.2.5 Plasma IL-8 concentration

Blood samples were collected as described in Chapter 2.4. Circulating levels of IL-8 were measured by enzyme-linked immunosorbent assay (ELISA) (Cusabio Biotech Co., Ltd) in plasma generated by centrifugation of whole blood for 5 minutes at 1500 g and 4°C, as described in Chapters 2.5.3 & 2.5.4.

### 5.2.6 Assessment of granulocyte adhesion and activation by flow cytometry

Flow cytometry was used to evaluate changes in CD11b and CD62L expression of granuloctyes in whole blood. Cells were stained with fluorochrome-conjugated antibodies specific for CD11b (10 µg/ml) and CD62L (0.1 mg/ml). An isotype IgG2a (10 µg) was used as a negative control. To assess granulocyte functions after stimulation, whole blood was stimulated with 1 µg formyl-Met-Leu-Phe-OH (fMLP). All data was collected on a FACS-Calibur flow cytometer and analysis performed using FlowJo Data Analysis Software. Please refer to Chapter 2.10 for more details.

### 5.2.7 Histology

Sections were stained with haematoxylin and eosin (H & E) to allow for identification and quantification of neutrophils (cells containing distinctive polymorphonuclear morphology) in the left ventricle, as described in Chapter 2.9.2.

### 5.2.8 2, 3, 5-triphenyltetrazolium chloride (TTC) staining

TTC staining was used to assess myocardial viability as described in Chapter 2.9.1. In some studies, in hearts from DEP-instilled rats, Evans blue was unable to discriminate between perfused and non-perfused myocardium due to increased capillary leak. In these studies, infarct size was expressed as a percentage of left ventricular (LV) mass rather than as a percentage of the area at risk (AAR).

### 5.2.9 Statistics

All values are expressed as mean ± SEM. Arrhythmias were clearly indicated by an abnormal change in blood pressure. The arrhythmia duration was defined as the total number of minutes rats exhibited irregular blood pressure. Relative radical concentrations (in arbitrary units, AU) were recorded as the signal intensity obtained from the peak-to-peak height of the first derivative EPR spectrum. Baseline systemic haemodynamics, plasma IL-8 concentrations, the adhesion and activation of granulocytes, myocardial AAR and infarct size were compared between the groups using one-way ANOVA combined with Bonferroni's post hoc test for multiple comparisons. Differences in haemodynamic parameters over time within and between groups were analysed by two-way ANOVA followed by Bonferroni post hoc test. An unpaired t-test was used to compare oxygen free radical generation. $P<0.05$ was considered to be statistically significant.

## 5.3 Results

### 5.3.1 I/R *in vivo*

#### 5.3.1.1 Effect of DEP instillation on resting heart rate (HR), blood pressure and rate pressure product (RPP)

No differences were observed in the resting HR of rats receiving no instillation or those 6 hours following instillation of saline or DEP (355 ± 12, 348 ± 13 and 377 ± 13 bpm respectively). Neither systolic nor diastolic arterial blood pressures under resting conditions were different 6 hours after saline instillation to that in rats that had no instillation (Table 5.1). Systolic and diastolic blood pressures were significantly higher in rats anaesthetised 6 hours after the instillation of DEP as compared to saline (Table 5.1). The RPP (HR multiplied by systolic arterial pressure) was calculated to provide an indication of myocardial demand. There was no difference in RPP in rats that received saline instillation compared to non-instilled rats (Table 5.1). However, RPP was significantly higher in rats 6 hours after exposure to DEP ($P<0.05$ compared to saline). Values reported here are consistent with those previously reported in Chapter 4.3.7.

#### 5.3.1.2 Effect of DEP instillation on HR, blood pressure and RPP during ischaemia and reperfusion *in vivo*

Induction of ischaemia by coronary artery ligation resulted in a transient drop in mean arterial blood pressure (MAP) in all groups (Table 5.2). This had returned to pre-ischaemic levels within 10-15 min for the non-instilled and saline-instilled animals while MAP in the DEP-instilled group continued to remain lower throughout the period of I/R (Table 5.2). In all groups there was a small (7-12%), statistically insignificant increase in the RPP at the onset of ischaemia. Thereafter, RPP remained relatively constant for the non-instilled and saline-instilled rats (Table 5.2). In the DEP-instilled rats RPP was significantly lower than baseline after 2 hour reperfusion (Table 5.2).

| | Group | | |
|---|---|---|---|
| | Non-instilled | Saline | DEP |
| HR (beats/min) | 355 ± 12 | 358 ± 10 | 389 ± 13 |
| SBP (mmHg) | 113.0 ± 7.4 | 100.7 ± 7.8 | **148.5 ± 5.2*** ** |
| DBP (mmHg) | 101.8 ± 8.1 | 89.6 ± 5.7 | **139.3 ± 5.2*** ** |
| RPP (HR X SBP/1000) | 31.2 ± 3.1 | 32.4 ± 1.8 | **41.9 ± 2.5*** |

**Table 5.1 Baseline cardiac parameters in non-instilled, saline-instilled and DEP-instilled rats.**

Abbreviations: DEP = diesel exhaust particulate, HR = heart rate, SBP = systolic blood pressure, DBP = diastolic blood pressure, RPP = rate pressure product.

Data are expressed as mean ± SEM (n=6). ***P<0.001 compared to saline; one-way ANOVA with Bonferroni post-hoc test.

| | Time | | | | |
|---|---|---|---|---|---|
| | Baseline | Onset Isc | 45 min Isc | 1 h Rep | 2 h Rep |
| **Non-instilled (n=6)** | | | | | |
| HR (beats/min) | 355 ± 12 | 341 ± 17 | 324 ± 14 | 330 ± 19 | 321 ± 20 |
| MAP (mmHg) | 105.5 ± 7.8 | **71.0 ± 2.6**$^{\#\#}$ | 94.3 ± 4.0 | 90.7 ± 2.4 | **76.7 ± 2.6**$^{\#\#}$ |
| RPP (HR X SBP/1000) | 31.3 ± 3.1 | 35.0 ± 1.0 | 30.5 ± 2.0 | 30.1 ± 1.6 | 24.7 ± 0.7 |
| **Saline (n=6)** | | | | | |
| HR (beats/min) | 358 ± 10 | 346 ± 10 | 335 ± 13 | 357 ± 7 | 342 ± 21 |
| MAP (mmHg) | 93.0 ± 6.4 | **59.4 ± 4.9**$^{\#\#\#}$ | 96.8 ± 6.6 | 91.5 ± 4.1 | 86.9 ± 5.2 |
| RPP (HR X SBP/1000) | 32.4 ± 1.8 | 34.8 ± 2.0 | 34.0 ± 4.1 | 33.1 ± 1.8 | 28.6 ± 2.3 |
| **DEP (n=4)** | | | | | |
| HR (beats/min) | 389± 13 | 365 ± 12 | 366 ± 16 | 367 ± 10 | 347 ± 14 |
| MAP (mmHg) | **140.5 ± 6.2*****| **82.7 ± 10.7**$^{\#\#\#}$ | **100.6 ± 8.5**$^{\#\#\#}$ | **89.8± 2.4**$^{\#\#\#}$ | **88.6 ± 2.8**$^{\#\#\#}$ |
| RPP (HR X SBP/1000) | **41.9 ± 2.5*****| 46.6 ± 6.8 | 38.1 ± 5.1 | 38.3 ± 5.4 | **30.2 ± 2.9**$^{\#}$ |

**Table 5.2 Cardiac parameters at timed intervals during ischaemia and reperfusion in non-instilled, saline-instilled and DEP-instilled rats.**

Abbreviations: DEP = diesel exhaust particulate, HR = heart rate, SBP = systolic blood pressure, MAP = mean arterial blood pressure, RPP = rate pressure product, Isc = ischaemia, Rep = reperfusion.

Data are expressed as mean ± SEM (n=4-6). ***P<0.001 versus saline; #P<0.05, ##P<0.01, ###P<0.001 versus baseline in each group; two-way ANOVA with Bonferroni post-hoc test.

#### 5.3.1.3 Effect of DEP instillation on cardiac arrhythmias during 45 minutes of ischaemia *in vivo*

Three needle electrodes were inserted subcutaneously into both hind-paws and the right fore-paw for electrocardiogram (ECG) measurements. No arrhythmias were seen during the baseline period in any of the experimental groups (representative traces are shown in Figures 5.1a & b). Characterising the specific types of arrhythmias was beyond the scope of this thesis. Arrhythmias were detected within 5 minutes of ischaemia induction (representative traces are shown in Figures 5.1c & d). In non-instilled rats these lasted for 7.6 ± 1.1 minutes (Figure 5.1e). Saline instillation did not modify arrhythmia duration (Figure 5.1e). In contrast, prior instillation of DEP significantly increased arrhythmia duration to 32.9 ± 5.0 minutes ($P = 0.0008$; Figure 5.1e). Additionally, in the DEP-instilled rats these arrhythmias were poorly tolerated; ventricular arrhythmias leading to sudden cardiac death within 15 minutes occurred in 60% (6 out of 10) of cases in the DEP-instilled group. Sudden cardiac death did not occur in the non-instilled and saline-instilled animals. There was no difference in the rate of deaths due to surgical- or anaesthetic-related mishaps.

#### 5.3.1.4 Effect of DEP instillation on myocardial infarct size after ischaemia and reperfusion *in vivo*

The AAR, determined after ligation was not different in rats that received saline instillation compared to non-instilled rats (Figure 5.2a). In DEP-instilled rats, initial studies indicated that Evans blue was not effectively excluded from the AAR indicating enhanced capillary leakage. Ultimately infarct size was calculated as a percentage of LV mass. Infarct size in non-instilled rats averaged 13.3 ± 1.0 % of total LV mass (Figure 5.2b) and was not changed by saline instillation. In contrast, infarct size in DEP-instilled rats was increased threefold compared with that in control rats ($P<0.001$).

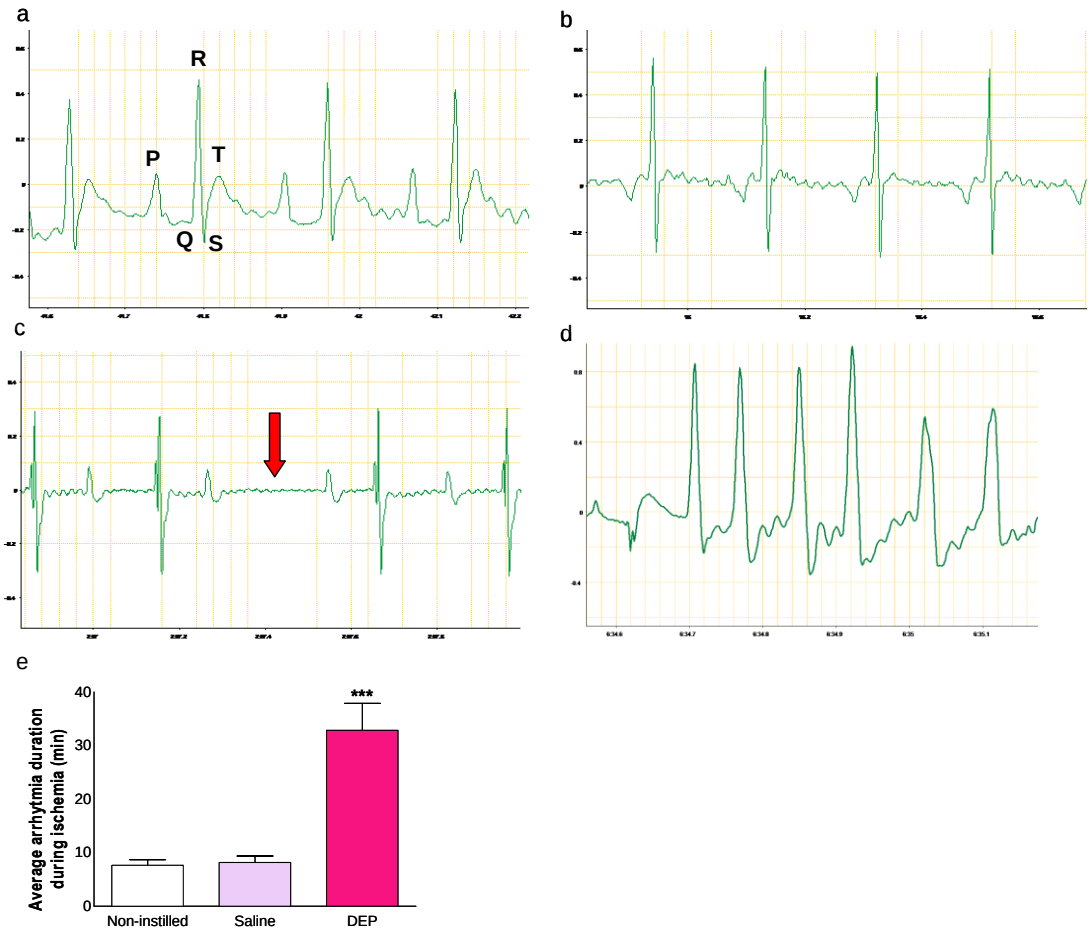


**Figure 5.1 Arrhythmic events increased during ischaemia in the *in vivo* rat heart 6 hours after instillation with diesel exhaust particulate (DEP).**

Representative traces of normal sinus rhythm showing standard PQRST waves from (a) saline- and (b) DEP-instilled rats at baseline. (c) Representative trace of a saline-instilled animal during ischaemia displaying 2$^{nd}$ degree atrioventricular (AV) block. (d) Representative trace of DEP-instilled rat during ischaemia displaying ventricular tachycardia (VT). (e) Duration of arrhythmias during ischaemia of surviving rats after no instillation, or 6 hours after instillation of saline or DEP. Columns represent mean ± SEM (n = 4-6) *** P<0.001, compared to saline; one-way ANOVA with Bonferroni post-hoc test.

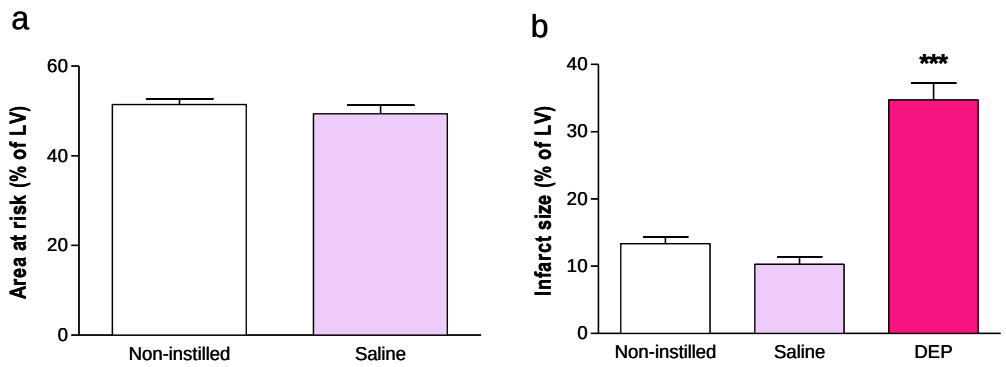


**Figure 5.2 Pulmonary exposure to diesel exhaust particulate (DEP) led to increased infarct size after *in vivo* ischaemia-reperfusion.**

(a) Area at risk (AAR; expressed as a percentage of total left ventricular (LV) mass) after no instillation and 6 hours after instillation of 0.5 ml saline. (b) Myocardial infarct size (expressed as a percentage of LV) after no instillation and 6 hours after instillation of saline or 0.5 mg DEP. Columns represent mean ± SEM (n = 4-6) *** $P<0.001$, compared to saline; one-way ANOVA followed by Bonferroni post-hoc test.

## 5.3.2 I/R *ex vivo*

### 5.3.2.1 Effect of DEP instillation on coronary perfusion pressure (CPP), AAR and infarct size after *ex vivo* I/R

The CPP (i.e the pressure at the coronary ostia) has been shown to be a major determinant of cardiac function (Broadley, 1979). Changes in CPP have been reported to affect functional changes of the hearts induced by I/R (Monasterio *et al.*, 2004). CPP was measured continuously at the aortic cannula using a transducer. There were no significant differences between the groups in baseline CPP (45.0 ± 6.1 ml/min for non-instilled, 48.1 ± 4.1 ml/min for 6 h saline and 52.5 ± 3.0 ml/min for 6 h DEP). There was a tendency towards increased perfusion pressure in all groups during the ischaemia and reperfusion, but the increase was not significant (Table 5.3). Changes in CPP during ischaemia and reperfusion were not different between groups.

The AAR was 73.3 ± 3.3 % in the non-instilled animals (Figure 5.3a). This was not further modified by instillation, regardless of whether saline or DEP was administered. Infarct size in hearts from non-instilled rats averaged 13.3 ± 1.0 % of total LV mass (Figure 5.3b) and was not changed in hearts from saline-instilled rats. In contrast, infarct size in hearts from DEP-instilled rats was increased threefold compared with that in control rats ($P<0.001$).

### 5.3.2.2 Effect of DEP instillation on oxygen free radical production at baseline and during the ischaemia and reperfusion in the isolated perfused rat heart

Baseline oxidant stress, determined by EPR in heart perfusate, 6 hours after instillation of saline was 2140.0 ± 224.0 AU (Figure 5.4a). Free radical intensity was approximately 3-fold higher in hearts from DEP-instilled rats ($P = 0.0035$; Figure 5.4a). All hearts showed a small burst of oxidant stress on reperfusion but this did not differ between treatment groups (Figure 5.4b). Unfortunately, there is no data available at this time on the free radical production in hearts from non-instilled rats due to time constraints.

#### 5.3.2.3 Effect of DEP instillation on wet weight and wet-to-dry weight ratio after *ex vivo* I/R

Wet heart weight, but not dry heart weight, was significantly higher from DEP-instilled rats than those from animals instilled with saline (P = 0.0053; Figure 5.5a). No changes were detected in the ratio of heart wet-to-dry weights between groups (Figure 5.5b).

| | Coronary perfusion pressure (CPP) values (mmHg) | | | | |
|---|---|---|---|---|---|
| | Baseline | Onset Isc | 30 min Isc | 1 h Rep | 2 h Rep |
| Non-instilled | 45.0 ± 6.1 | 58.8 ± 10.0 | 62.4 ± 8.7 | 62.4 ± 9.8 | 70.7 ± 16.7 |
| Saline | 48.1 ± 4.1 | 54.4 ± 4.9 | 64.1 ± 15.3 | 60.0 ± 14.4 | 62.7 ± 14.8 |
| DEP | 52.5 ± 3.0 | 62.0 ± 7.5 | 63.7 ± 9.8 | 58.1 ± 9.6 | 51.8 ± 3.0 |

**Table 5.3 Coronary perfusion pressures sampled at various time intervals during ischaemia and reperfusion in hearts isolated from non-instilled, saline-instilled and DEP-instilled rats.**

Abbreviations: DEP = diesel exhaust particulate, Isc = ischaemia, Rep = reperfusion. Data are expressed as mean ± SEM (n=4-5).

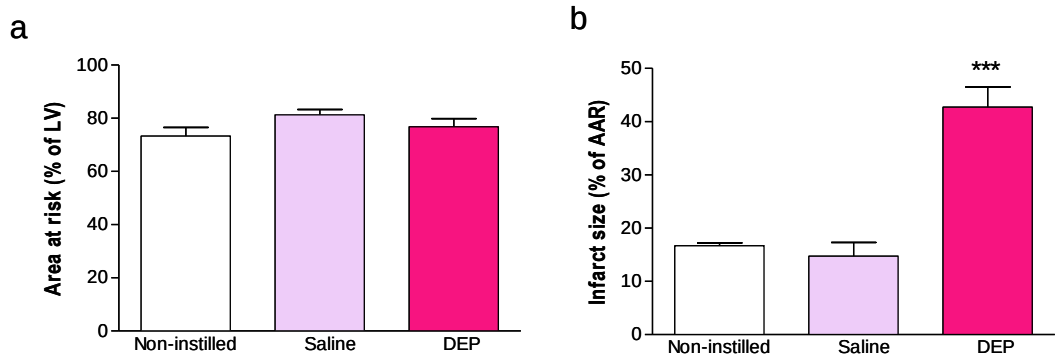


**Figure 5.3 Infarct size was bigger in Langendorff-perfused hearts from rats instilled with diesel exhaust particulate (DEP).**

(a) Area at risk (AAR; expressed as a percentage of total left ventricular (LV) mass) and (b) myocardial infarct size (expressed as percentage of AAR) in non-instilled rats and 6 hours after instillation of 0.5 ml saline or DEP (0.5 mg). Columns represent mean ± SEM (n = 4-6) *** P<0.001, compared to saline; one-way ANOVA followed by Bonferroni post-hoc test.

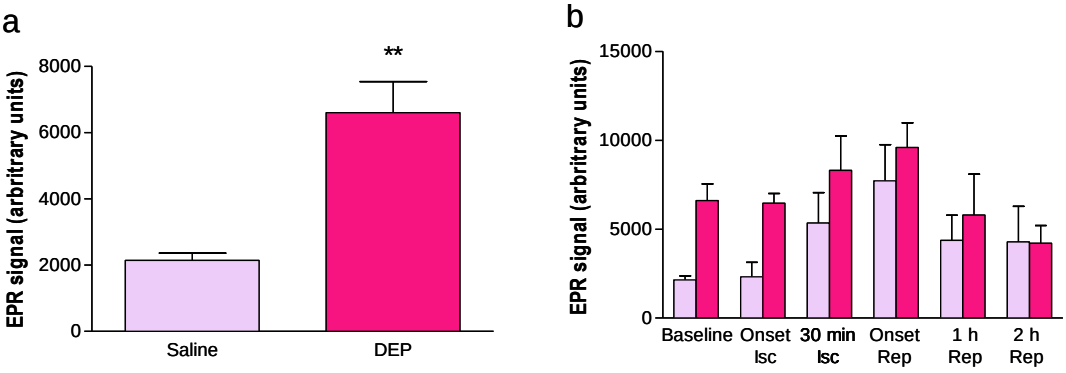


**Figure 5.4 At baseline oxygen free radical formation in perfused rat hearts was higher in animals instilled with diesel exhaust particulate (DEP).**

Electron paramagnetic resonance (EPR) signal of the 1-hydroxy-3-carboxy-pyrolidine (CP-H) spin adduct at (a) baseline and at (b) different times during the ischaemia/reperfusion (I/R) 6 hours after instillation of 0.5 ml saline or DEP (0.5 mg). Columns represent mean ± SEM (n = 4) ** P<0.01, compared to saline; unpaired t-test

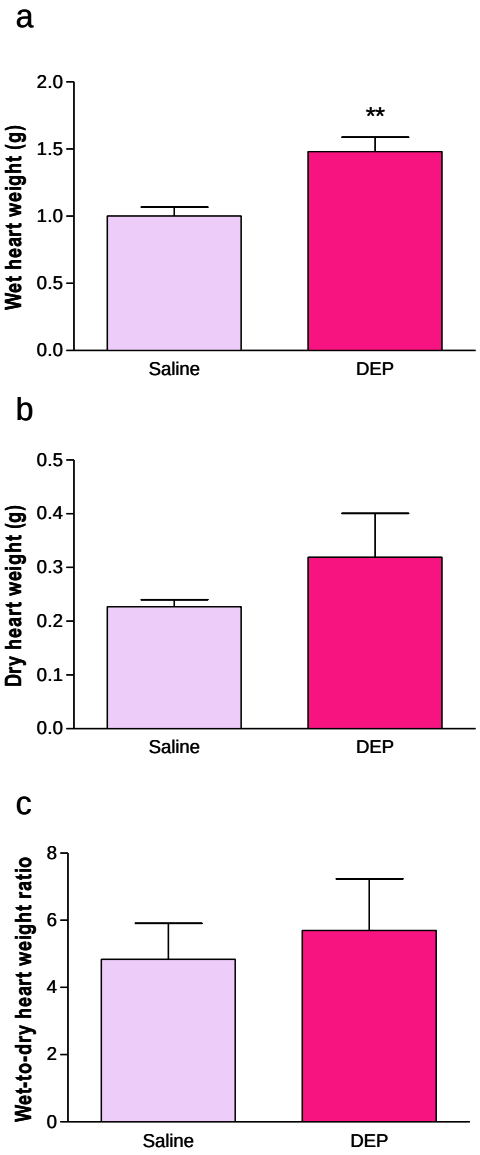


**Figure 5.5 After ischaemia/reperfusion (I/R) wet weight of the isolated rat hearts was higher in rats instilled with diesel exhaust particulate (DEP).**

(a) Wet heart weight, (b) dry weight and (c) wet-to-dry heart weight ratio 6 hours after instillation of 0.5 ml saline or DEP (0.5 mg). Columns represent mean ± SEM (n = 5) ** P<0.01, compared to saline; unpaired t-test.

### 5.3.3 Influence of DEP on tissue injury, cytokines and neutrophil activation state prior to I/R

#### 5.3.3.1 Effect of DEP instillation on heart wet weight

Before I/R wet heart weight was significantly higher from DEP-instilled rats than non-instilled animals (Figure 5.6).

#### 5.3.3.2 Effect of DEP instillation on cardiac cell viability

Staining of hearts with TTC was used to determine whether particle instillation led to irreversible myocardial damage. The LV had negligible levels of damage in hearts from non-instilled rats or at 6 hours following saline instillation. However, 10.1 ± 2.6% of the LV stained brick red with TTC (TTC-positive) in hearts collected 6 hours after instillation of DEP (Figure 5.7).

#### 5.3.3.3 Effect of DEP instillation on circulating IL-8 concentrations

Plasma IL-8 concentration in non-instilled rats was 266.3 ± 28.4 pg/ml (Figure 5.8) and this was not significantly altered 6 hours after saline or DEP instillation (Figure 5.8).

#### 5.3.3.4 Effect of DEP instillation on myocardial neutrophil tissue number

H & E stained left ventricle sections revealed no penetration of neutrophils into the cardiac tissue in any of the groups (Figure 5.9).

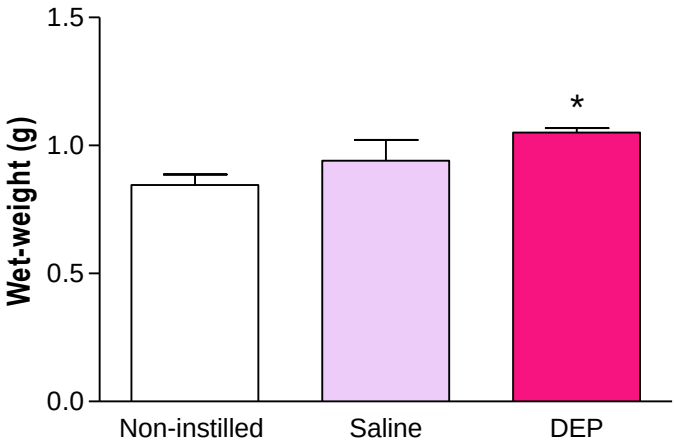


**Figure 5.6 Diesel exhaust particulate (DEP) instillation increased heart wet weight.**

Wet heart weight after no instillation and 6 hours after instillation of 0.5 ml saline or DEP (0.5 mg). Columns represent mean ± SEM (n = 4) * P<0.05, compared to non-instilled; one-way ANOVA with Bonferroni post-hoc test.

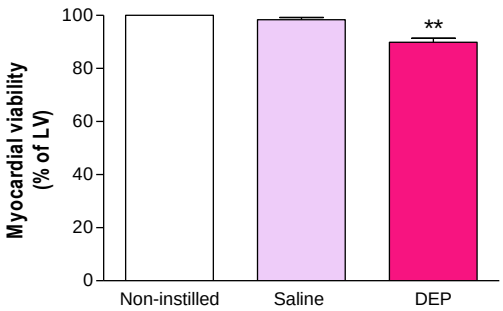


**Figure 5.7 Diesel exhaust particulate (DEP) instillation decreased cardiac cell viability.**

The percentage of viable myocardium in the left ventricle (LV) after no instillation and 6 hours after instillation of 0.5 ml saline or DEP (0.5 mg) was calculated by deducting the percentage of tissue stained brick with 2, 3, 5-tetrazolium chloride (TTC). Columns represent mean ± SEM (n = 3) ** P<0.01, compared to saline; one-way ANOVA with Bonderroni post-hoc test.

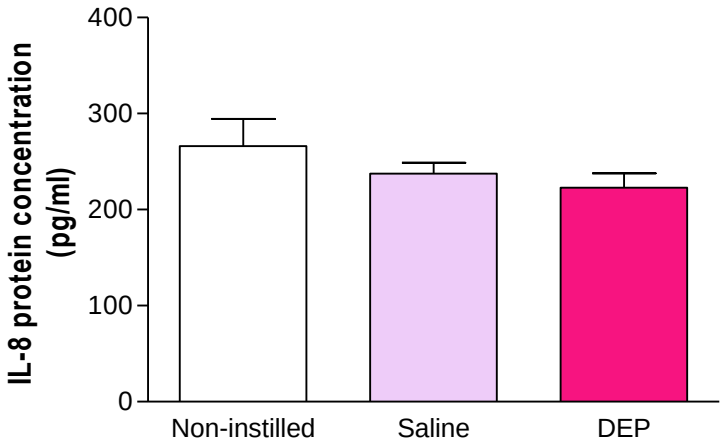


**Figure 5.8 Instillation of diesel exhaust particulate (DEP) does not affect plasma interleukin-8 (IL-8) concentrations.**

Plasma IL-8 concentrations in non-instilled, saline-instilled and DEP-instilled (0.5 mg) rats 6 hours after instillation. Plasma IL-8 concentrations were detected by enzyme-linked immunosorbent assay (ELISA). Columns represent mean ± SEM (n=4-5).

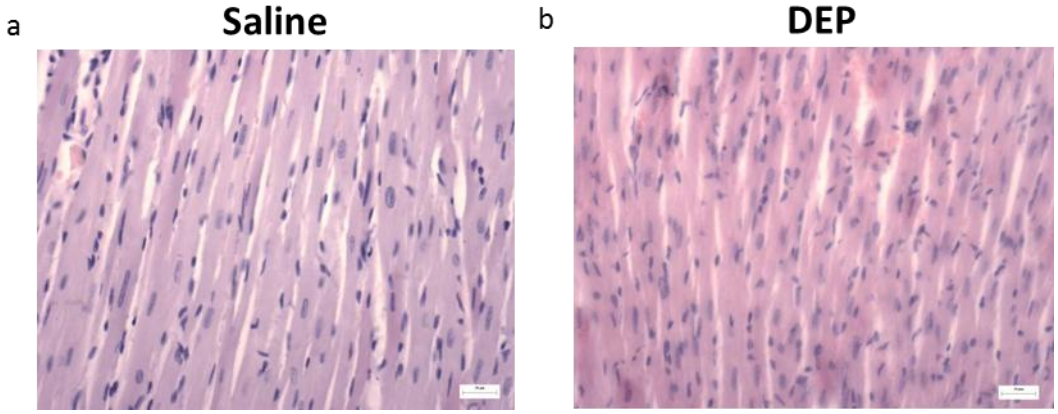


**Figure 5.9 No infiltrating neutrophils were found in rat heart 6 hours after pulmonary exposure to diesel exhaust particulate (DEP).**

Haematoxylin and eosin (H & E) stained sections of left ventricle (LV) 6 hours after instillation of (a) 0.5 ml saline or (b) 0.5 mg DEP. Scale is 25 µm.

#### 5.3.3.5 Effect of DEP instillation on whole blood flow cytometric analysis of granulocyte adhesion and activation

Granulocytes were analysed after exclusion of other cells (red blood cells (RBC), monocytes and lymphocytes) using forward and side scatter parameters. Two distinct sub-populations of CD11b expressing cells were identified, either low ($CD11b^{LOW}$) or high ($CD11b^{HIGH}$) median fluorescence intensity (MFI) (Figure 5.10). MFI calculated for $CD11b^{LOW}$ and $CD11b^{HIGH}$ in blood from non-instilled rats was 41.7 ± 4.8 and 238.3 ± 12.4 (arbitrary units) respectively (Figures 5.11a &c). The intensity of expression for $CD11b^{LOW}$ and $CD11b^{HIGH}$ populations were not altered in blood collected 6 hours after instillation of saline or DEP (Figures 5.11a & c). In blood from all treatment groups stimulation *ex vivo* with fMLP caused a decrease in the intensity of expression of the $CD11b^{LOW}$ populations (Figure 5.11b). In contrast, fMLP treatment of granulocytes in whole blood of non-instilled rats increased the intensity of expression of $CD11b^{HIGH}$ (Figure 5.11d). Neither saline nor DEP instillation altered the MFI for fMLP-stimulated $CD11b^{LOW}$ or $CD11b^{HIGH}$ granulocytes (Figure 5.11d). In addition, the ratio of $CD11b^{LOW}$/$CD11b^{HIGH}$ was not different between the groups (data not shown). In all groups no CD62L expressing cells were detected above the levels of background staining seen in the isotype controls.

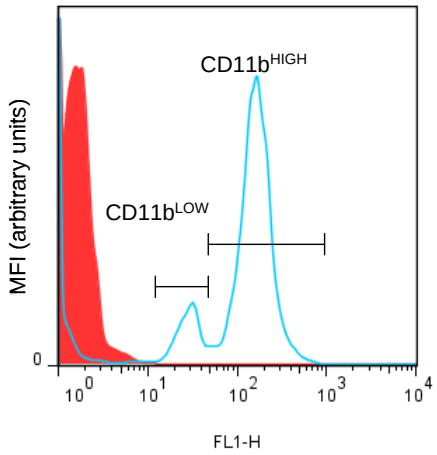


**Figure 5.10 Flow cytometric analysis of granulocyte CD11b expression.**

Representative fluorescence histogram profile of normal rat peripheral blood granulocytes stained with CD11b antibody. The histogram shows a clear bimodal distribution, with $CD11b^{LOW}$ and $CD11b^{HIGH}$ populations readily discernible. The median fluorescence intensity (MFI) measured on a logarithmic scale (in arbitrary units) was calculated for each peak (seen as a bracketed horizontal line). The red peak represents the isotype control.

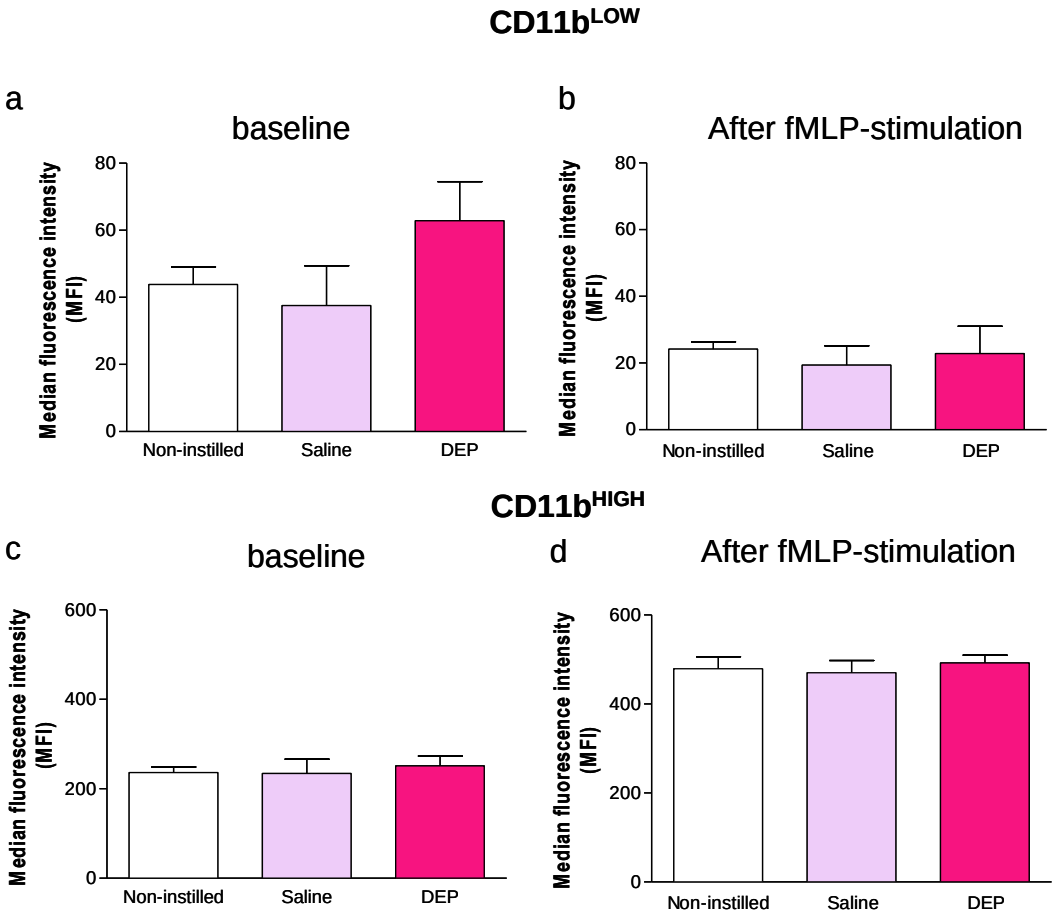


**Figure 5.11 Diesel exhaust particulate (DEP) instillation did not change expression of CD11b$^{LOW}$ and CD11b$^{HIGH}$ granulocytes at baseline or after *ex vivo* stimulation.**

Median fluorescence intensity (MFI) for CD11b$^{LOW}$ and CD11b$^{HIGH}$ granulocyte subsets in non-instilled rats and 6 hours after instillation of saline or DEP (0.5 mg) at baseline (a & c) and following 1 µM formyl-Met-Leu-Phe-OH (fMLP) stimulation (b & d). Columns represent mean ± SEM (n = 4-6)

## 5.4 Discussion

The data presented here are consistent with the hypothesis that instillation of DEP into the lungs of rats increases the vulnerability of the heart to subsequent myocardial reperfusion injury. These studies have also shown that a single exposure of the lung to DEP primes the heart making it more susceptible to subsequent ischaemic injury within as little as 6 hours following exposure.

### 5.4.1 *In vivo* studies

There is strong epidemiological, clinical and experimental data demonstrating associations between increased particulate air pollution and the triggering of arrhythmias(Dockery *et al.*, 2005; Hazari *et al.*, 2011; He *et al.*, 2010; Liao *et al.*, 2010; Peters *et al.*, 2000; Watkinson *et al.*, 1998; Wellenius *et al.*, 2002). Watkinson *et al.* (1998) reported increased cardiac arrhythmia after a single intra-tracheal instillation of residual oil fly ash (ROFA) in normal rats. Without a regular ECG assessment we could not characterise the type of cardiac arrhythmia. Nevertheless, in the current study, the total duration of arrhythmias during 45 minutes of ischaemia was significantly longer in the DEP-instilled rats. The increased susceptibility to arrhythmias was associated with an increased oxygen demand during ischaemia (higher RPP). This could be a reason for increased injury as increased demand would mean more damage when supply is limited. It was also observed that a single exposure to DEP increased I/R-related mortality. These observations point towards the ability of a single exposure to DEP to increase the vulnerability of the heart to developing arrhythmia caused by subsequent ischaemia and reperfusion. Regarding DEP, Yokota *et al.* (2004) previously showed exacerbation of ventricular arrhythmias during the reperfusion period and increased reperfusion-related mortality 24 to 48 hours after instillation of 1 mg DEP. These investigators concluded that this increased susceptibility was due to increased generation of ROS by recruited neutrophils (Yokota *et al.*, 2004; Yokota *et al.*, 2005).

It is well recognised that circulating neutrophils play an important role in myocardial I/R injury (Jordan *et al.*, 1999). Indeed, neutrophils migrate from the circulation into the ischaemic tissue via endothelial cell interactions and produce an inflammatory response within the first few minutes of reperfusion (Yellon *et al.*, 2007). The study in Chapter 4 of this thesis showed an increased circulating white blood cell (WBC) count 6 hours after instillation of DEP. One explanation for the increased infarct size after DEP instillation might be increased recruitment of neutrophils into ischaemic myocardium. This is supported by the finding that increased infarct size in animals instilled with CAP 24 hours earlier was positively associated with increased myocardial neutrophil tissue number (Cozzi *et al.*, 2006). Prior activation or priming of neutrophils in DEP-instilled rats might also enhance their recruitment and also their subsequent activation and contribution to injury. Thus, it was considered worthwhile to evaluate the effect of DEP instillation on the surface expression of CD11b on granulocyte cells. Under resting conditions, the CD11b subunit of the granulocyte adhesion molecule (β2 integrins), is mainly stored within intracellular granules (Mazzone *et al.*, 1995). CD11b surface expression on granuloctyes rapidly increases upon activation leading to their sequestration and adhesion to the endothelium. Thereafter, activated granulocytes release ROS. In the current study, there was no change in the surface expression of granulocytes 6 hours after instillation of DEP, indicating that pulmonary DEP did not influence the activation levels of systemic leukocytes. Instillation of DEP also had no effect on CD11b expression of granulocytes primed with fMLP, suggesting that DEP instillation does not prime systemic granulocytes causing heightened response during ischaemia and reperfusion. IL-8 is a potent chemotactic factor for neutrophils (Ghelfi *et al.*, 2008). Plasma concentrations of IL-8 did not change after DEP instillation compared with rats treated with saline instillation. This result suggests that IL-8 did not contribute towards the increased circulating WBC count 6 hours after instillation of DEP (see Chapter 4). Similarly the plasma levels of another stimulator of neutrophils, tumour necrosis factor alpha (TNFα) (Benbarek *et al.*, 2008) were not found to differ between groups in studies described in Chapter 4. Other inflammatory mediators known to be important for the mobilisation of leukocytes include interleukin-1 beta (IL-1β), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage

colony stimulating factor (GM-CSF) (Springer, 1994) and warrant further investigation. In line with our study, no effect on CD11b expression was also observed by Cozzi *et al.* (2005) 24 hours after a single instillation of CAP in mice.

It cannot be completely ruled out that the increased susceptibility to ischaemia-induced arrhythmias was not due to a larger ischaemic zone in the DEP-instilled animals (Curtis, 1998). In hearts from DEP-instilled rats, Evans blue could not accurately distinguish perfused from non-perfused tissue after *in vivo* I/R. Upon visual examination the cardiac tissue from the DEP-instilled rats showed blue staining throughout the heart. Assessment of AAR is important as the size of the ischaemic zone is the most important determinant of ultimate infarct size (Lowe *et al.*, 1978). If by chance the AAR was higher in hearts from DEP-instilled rats, then this alone could explain the increased infarct size. However, this seems unlikely to be the case here since in the *ex vivo* I/R studies (discussed in detail below) the AAR was not different between groups but increased infarct size was still observed. Also, there was reason to believe that DEP instillation caused microvascular leakage, thereby preventing discrimination between non- and ischaemic tissue. The wet heart weight before I/R was significantly higher in those rats instilled with DEP, supporting the presence of oedema. Gurgueira and co-workers reported oedema in the hearts of rats exposed to ambient PM *in vivo* (Gurgueira *et al.*, 2002). These investigators suggested that the cardiac oedema was due to an increased generation of ROS. In the current study, more ROS were generated *ex vivo* in the hearts from DEP-instilled rats and this will be discussed in more detail below. Systemic inflammation may also lead to oedema (Martin *et al.*, 1988). However, this seems unlikely, given that studies in Chapter 4 of this thesis did not detect increased circulating levels of pro-inflammatory cytokines 6 hours after the instillation of DEP. Since Evans blue could successfully identify the AAR in the *ex vivo* I/R studies, it is possible that neural influences in response to DEP exposure are altering microvascular permeability. Further experiments are warranted to confirm whether DEP-instillation increases microvascular permeability and capillary leak, which in turn results in oedema using Evans blue extravasation.

Another possible mechanism is that DEP alters the autonomic nervous system (ANS) and increases the arrhythmogenic response to ischaemia and reperfusion. Both human and animal studies have reported associations between PM and triggering of ANS reflexes, eventually leading to arrhythmia (Godleski *et al.*, 2000; Peters *et al.*, 2000; Wellenius *et al.*, 2002). Decreased heart rate variability (HRV, a marker of cardiac sympathetic activation) and increased incidence of arrhythmia have been reported in rats exposed to ROFA (Wellenius *et al.*, 2002). Ceremuzynski *et al.* (1969) reported that increased plasma catecholamine concentrations in dogs correlated directly with the severity of ventricular arrhythmias during ischaemia, lending further support to this concept. Plasma catecholamine concentrations have been notoriously difficult to measure (Boomsma *et al.*, 1993; Goldstein, 2003) and studies described in Chapter 4 of this thesis could not detect elevated catecholamine levels in the plasma of DEP-instilled rats. Nevertheless, in the current study, DEP instillation caused an increase in both baseline SBP and DBP which would be consistent with increased sympathetic activation. An increased sympathetic activation would likely explain the myocardial hypersensitivity to ischaemia-induced arrhythmias in the DEP-instilled rats. Activation of the sympathetic nervous system (SNS) may also explain the increased irreversible myocardial damage in these animals (Rona, 1985). The mechanisms by which SNS may induce myocardial cell damage is likely multifactorial, including ROS and $Ca^{2+}$ overload (Rona, 1985).

In summary, the data presented here showed that rats receiving a single exposure of DEP developed larger infarcts following I/R *in vivo*. In addition, these rats were more susceptible to ischemia-induced arrhythmias. Experiments using flow cytometry showed this increased reperfusion injury in the DEP-instilled rats was not due to prior neutrophil activation. Studies in Chapter 4 of this thesis also detected no systemic cytokine concentration after DEP instillation. However, the *in vivo* experiments do not allow us to determine whether the increase in infarct size is dependent on enhanced neutrophil recruitment or on other neurohormonal responses to I/R *in vivo*.

### 5.4.2 *Ex vivo* studies

To determine the dependence of increased injury on neurohormonal influences *in vivo* and on potentially altered inflammatory cell recruitment from the blood, studies were therefore also performed *ex vivo* on hearts from saline- and DEP-instilled rats. An increase in infarct size after I/R was observed in hearts from rats instilled with DEP 6 hours previously and then buffer-perfused *ex vivo*. It was also found, that at equivalent flow rates, CPP at baseline and during I/R did not differ between the DEP- and saline-instilled groups. These findings suggest that the increase in infarct size in DEP-instilled rats was not, therefore, due to alterations in coronary endothelial function (Broadley, 1979), although further studies would be necessary to confirm this. Studies in Chapter 4 of this thesis demonstrated that endothelium-dependent vasodilation was not altered in the peripheral vascular bed of DEP-instilled rats. Also abnormal endothelial cell function can contribute to variations in the myocardial AAR. In the present study, AAR did not differ between groups, showing that the increased susceptibility to reperfusion injury was not due to a larger ischaemic zone in the DEP-instilled group.

In the current study instillation with DEP increased infarct size in the absence of neural or haemodynamic factors. Together with the *in vivo* data, these data strongly suggest that instillation of DEP causes *in vivo* priming of hearts to subsequent injury.

### 5.4.3 Implications of oxidative stress

Possible mechanism(s) responsible for priming of the heart for subsequent injury were explored next. Oxidative stress can have damaging effects on the heart. ROS target all major cellular components, including proteins and lipids. Excess production of ROS may ultimately lead to arrhythmia, contractile failure and cell death (Guo *et al.*, 2005; Kevin *et al.*, 2005; Kim *et al.*, 2006; Shattock *et al.*, 1991; Yu, 1994). In the current study, oxygen-centred free radical generation from the *ex vivo* retrograde perfused heart was measured using EPR spectroscopy coupled with CP-H spin-trapping. More ROS were detected in the coronary effluent of hearts from the DEP-instilled rats. Previous studies of animals exposed to PM have

demonstrated the occurrence of oxidative stress in the heart (Gurgueira *et al.*, 2002; Tankersley *et al.*, 2008). In the present study, it remains unclear whether this is due to increased ROS production and/or a reduced level of activity of anti-oxidants. There were no infiltrating neutrophils detectable on H & E-stained paraffin sections from hearts of rats instilled with DEP 6 hours previously. Thus, it is unlikely that neutrophils are a major source of the excess ROS in these hearts. Other potential sources of the ROS in the myocardium include mitochondria, xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Jordan *et al.*, 1999). Coronary arterioles from rats exposed to DE increased ROS generation through nitric oxide synthase (NOS) uncoupling (Cherng *et al.*, 2009). *In vitro* studies on alveolar macrophages exposed to DEP implicated the mitochondria as a major source of the ROS (Hiura *et al.*, 2000). Mo and colleagues, on the other hand found that rotenone, a mitochondrial inhibitor, did not alter DEP-induced ROS generation in mouse pulmonary microvascular endothelial cells (MPMVEC) (Mo *et al.*, 2009). These investigators showed the formation of oxygen-derived free radicals by the DEP to be catalysed by a membrane-bound NADPH oxidase. Supporting evidence for the involvement of NADPH oxidase has recently been obtained in an animal inhalation study using PM (Kampfrath *et al.*, 2011). NADPH oxidases are of particular interest since these enzymes are widely distributed within heart cells (Cave *et al.*, 2005). In this regard, future studies using inhibitors of ROS-producing systems will be insightful. Future studies should also include measurements of anti-oxidant enzyme activities using spectrophotometric methods. The effect of exposure on anti-oxidant enzyme activities has been studied with conflicting results. Anti-oxidant enzyme activity was significantly inhibited *in vitro* in rat ventricular cardiac myocytes treated with DEP (Okayama *et al.*, 2006). Others have shown decreasing anti-oxidant enzyme activity in the heart of rats after inhalation of carbon monoxide (CO) (Andre *et al.*; Meyer *et al.*, 2010) In contrast, there are reports that PM exposure generates an adaptive response by activating anti-oxidant defences (Gurgueira *et al.*, 2002).

At present little is known of the molecular mechanisms underlying activation of the ROS-generating enzymes by PM. This might be related to the activation of pro-inflammatory cytokines which in turn can promote the activation of ROS-generating

enzymes (Nakamura *et al.*, 1998). However, as discussed above this seems unlikely to have contributed to the cardiac oxidative stress in this model given that no systemic neutrophil or pro-inflammatory cytokine activation was detected. Studies in the literature suggest that PM exposure might increase cardiac oxidants via autonomic activation (Ghelfi *et al.*, 2008; Rhoden *et al.*, 2005). Rhoden and colleagues demonstrated in rats, that inhalation of CAP induced cardiac oxidative stress that was abolished by sympathetic and parasympathetic antagonists (Rhoden *et al.*, 2005). These investigators also showed that the increased incidence of spontaneous cardiac arrhythmias in CAP-exposed rats could be prevented by treatment with the free radical scavenger superoxide dismutase (SOD).

Regardless of the mechanism, however, we have demonstrated that instillation of DEP leads to increased oxidant levels in the rat heart. Hearts from DEP-instilled rats also had an increased area of myocardium with reduced viability, assessed by TTC and also an increased number of apoptotic cells (see Appendices 3 & 4). It seems highly likely that this is related to the increased oxidant stress. Okayama *et al.* (2006) found that DEP significantly decreased rat myocardial cell viability *in vitro*. This was prevented by co-incubation with anti-oxidant enzymes, including SOD and catalase. In this study the authors made the assumption that the DEP can definitely translocate to extra-pulmonary tissues. However, experimental evidence supporting the translocation of PM is still lacking and urges the need for additional studies to evaluate the role of oxidative stress in mediating cell death induced by DEP. Future studies will, therefore, determine whether anti-oxidants abrogate the apoptosis induced by DEP instillation. Furthermore, it seems likely that these phenotypic changes increase the severity of injuries related to I/R. The current study may also help to explain why PM increased the I/R-induced infarct size but did not alter redox status 24 hours after instillation (Cozzi *et al.*, 2006). Since earlier times were not examined by these investigators it remains possible that the oxidative stress had reversed by 24 hours. However, it is unlikely that the process of apoptosis would be reversed by this time which might explain the increased infarct size.

Although not assessed in this study it is possible that DEP is inducing apoptosis via elevated intracellular calcium ($Ca^{2+}$). Increased intracellular $Ca^{2+}$ concentrations have been reported in myocardial cells cultured and treated with ambient particles (Bagate *et al.*, 2006a). *In vitro* experiments have also shown PM to induce apoptosis in human epithelial cells lining the airways through an increase in $Ca^{2+}$ intracellular levels (Agopyan *et al.*, 2004). In addition to cell death alterations in $Ca^{2+}$ homeostasis contribute to both arrythmogenesis (Kihara *et al.*, 1991; Zaugg *et al.*, 1996) and contractile dysfunction (Liu *et al.*, 2008). Alterations in $Ca^{2+}$ homeostasis may also help to explain the increased arrhythmias observed during ischaemia 6 hours after DEP instillation. It can also not be ruled out that the elevated oxygen free radicals are increasing $Ca^{2+}$ by damaging the proteins involved in $Ca^{2+}$ handling. Future experiments will be needed to determine wither DEP instillation influences intracellular $Ca^{2+}$ in rat myocytes using fluorescence microscopy. Finally, it is worth noting that in the current study the RPP was higher 6 hours after instillation with DEP. It may be that the delivery of oxygen to the heart may be obstructed sufficiently by the pulmonary inflammation in these animals (please refer to Chapter 4) to cause cell death.

It is well established that there is a burst of oxygen free radical generation in the heart at the time of post-ischaemic reperfusion (Zweier *et al.*, 2006). In the present study, all hearts showed a small burst of oxidant stress on reperfusion but this did not differ between treatment groups. The oxygen radicals generated are reported to play a pathogenetic role in arrhythmias and cell death (Bolli, 1991). The results of the present study clearly indicate free radical production from non-neutrophil sources but the exact ROS source is as yet unknown. Notably, in the present study there was no significant difference in the percentage increase in free radicals upon re-introduction of oxygen between the groups. It therefore seems unlikely that the increased susceptibility to reperfusion injury in the hearts from the DEP-instilled animals is associated with the free radicals generated during the ischaemia and reperfusion. This demonstration that free radical generation during I/R was not different between groups may also suggest that the increased susceptibility to reperfusion injury in

hearts from DEP-instilled animals was not due to compromised cellular anti-oxidant capacity.

### 5.4.4 Direct effects of DEP on the heart

A direct effect of translocated DEP (or their associated constituents) cannot be entirely ruled out. While some studies have demonstrated the translocation of nanoparticles from the lung to the systemic circulation (Brown *et al.*, 2002; Kreyling *et al.*, 2002; Oberdorster *et al.*, 2004) there remains considerable uncertainty over whether this mechanism underlies the health effects of combustion-derived nanoparticles (Brook *et al.*, 2004; Mills *et al.*, 2006). Using ultrafine iridium particles, studies by Kreylling *et al.* (2002) reported minimal (<1%) translocation of particles deposited in the lung to secondary target organs one week after a 1 hour exposure (Kreyling *et al.*, 2002). Therefore, if DEP did translocate from the lung it seems unlikely that particles would account for the full biological effects of DEP entering the lung.

### 5.4.5 Limitations

Several limitations need to be considered. As discussed above, an *ex vivo* I/R model was employed to evaluate neurohormonal influences on the increased myocardial injury. In these experiments, hearts were perfused at constant flow opposed to constant pressure. While this allows easy measurement, it does not automatically alter the amount of perfusate delivered to the whole heart in response to ischaemia (Skrzypiec-Spring *et al.*, 2007).

Amongst other factors, variations in temperature have been reported to influence myocardial contractile responsiveness (Skrzypiec-Spring *et al.*, 2007). In this study hearts were enclosed in a sealed, water-jacketed chamber to maintain temperature at 37 +/- 0.5 °C. Other problems known to affect cardiac performance come from contamination of the reagent solutions and glassware. For this reason, fresh buffers were prepared and the perfusion apparatus thoroughly cleaned after use. Precautions were also taken to preserve hearts from ischaemic injury prior to cannulation by

immediately placing the heart in ice cold perfusate following excision to temporally arrest its motion. Care was also taken to ensure that no air bubbles were found in the circuit as these may lead to significant ischaemic damage. Additionally, anticoagulation by heparin administration was performed to minimise risk of thrombi formation in the excised heart.

Future studies should also include ECG diagnostic criteria and longer-term assessment in both *in vivo* and *ex vivo* I/R models. Comparing arrhythmias during *in vivo* and *ex vivo* electrophysiological testing will better define the mechanism for the increased propensity to ischaemia-induced arrhythmias in the hearts of the DEP-instilled animals.

### 5.4.6 Conclusions

The data presented here suggest a single exposure of the lung to DEP primes the heart making it more susceptible to subsequent myocardial injury within as little as 6 hours following exposure. This may help to explain recent clinical observations that have identified this as a key period for increased myocardial mortality after exposure to PM (Bhaskaran *et al.*, 2011). This increased reperfusion injury was characterised by increased propensity to arrhythmia development during ischaemia and also to increased infarct size. In these studies, instillation of DEP by itself was shown to induce oxidative stress and cell loss, at least in part due to increased apoptosis. These phenotypic changes may explain the effects of exposure to DEP on I/R-induced injury. Studies in Chapter 6 of this thesis will begin to examine how DEP initially deposited in the lung might influence injury in the myocardium, with particular emphasis on the ANS. As discussed above, disturbances in ANS function may be related to arrhythmia, increased oxidative stress and cell death.

# Chapter 6

# Potential role of transient receptor potential vanilloid 1 (TRPV1) receptor and (beta 1) $\beta_1$ adrenoceptor activation on the transmission of the influence of diesel exhaust particulate (DEP) from the lung to the myocardium

## 6.1 Introduction

The data in Chapter 5 of this thesis clearly demonstrated that a single exposure of the lung to DEP leads to priming of the myocardium for ischaemia-reperfusion (I/R) injury. Candidates for transmission of the effects of DEP in the lung to the periphery include systemic inflammation (Seaton *et al.*, 1995) and alteration of autonomic outflow (Pope *et al.*, 2004; Pope *et al.*, 1999). Earlier studies described in this thesis detected no systemic inflammation 6 hours after intra-tracheal instillation of DEP. It thus seems unlikely that the damaging effects to the myocardium caused by DEP instillation are driven by systemic inflammation. Interestingly, studies in Chapter 5 did produce findings that would be consistent with sympathetic activation 6 hours after DEP instillation;-the most notable being increased blood pressure and increased incidence of ischaemia-induced arrhythmias. Increased sympathetic activity is also consistent with previous human and animal studies reporting associations between air pollutants and decreased heart rate variability (HRV) (Brook *et al.*, 2009a; Gold *et al.*, 1999; Pope *et al.*, 1999; Rivero *et al.*, 2005; Schulz *et al.*, 2005). Likewise, heightened arrhythmia sensitivity and cardiac oxidative stress and injury have been reported to be mediated by autonomic stimulation following exposure to residual oil fly ash (ROFA) or concentrated ambient particles (CAPs) (Rhoden *et al.*, 2005; Wellenius *et al.*, 2002).

The sympathetic adrenergic control of the heart originates from neurons found within the medulla. The central nervous system (CNS) receives sensory (afferent) input from peripheral sensors and sensors within the brain. The majority of afferent fibres innervating the lung are non-myelinated C-fibres (Coleridge *et al.*, 1984). These fibres innervate sensory receptors and transmit information from the airways to the nucleus tractus solitarius (NTS) through the vagus nerve. Irritant air pollutants such as ozone (Lee *et al.*, 2001), sulphur dioxide ($SO_2$) (Wang *et al.*, 1996), wood smoke (Lai *et al.*, 1998) and diesel exhaust (DE) (Wong *et al.*, 2003) have all been shown to stimulate pulmonary C-fibre afferents. TRVP1, a non-selective cation channel, is widely expressed in these sensory nerve fibres (Watanabe *et al.*, 2005). Activation of TRPV1 expressed in pulmonary nerve terminals by both physical and chemical

stimuli leads to the release of substance P (SP) and calcitonin gene-related peptide (CGRP) (Bevan *et al.*, 1994). Moreover, it has been hypothesised that TRPV1 activation on non-myelinated C-fibres mediates powerful autonomic reflexes which produce changes in the cardiovascular function (Widdicombe *et al.*, 2001). Whether stimulation of TRPV1 receptors provides a plausible link between pulmonary DEP and injury in distal tissues constitutes the focus of the present study. To date, only one previous study has explored the association between pollution-related cardiac toxicities and TRPV1 stimulation (Ghelfi *et al.*, 2008).

### 6.1.1 Hypothesis

Pulmonary instillation of DEP leads to priming of the myocardium for I/R injury via activation of pulmonary TRPV1 receptors and systemic $\beta_1$ adrenoceptors.

### 6.1.2 Aims

The aims of the work described in this chapter were to determine whether:

i. $\beta_1$ adrenoceptor and TRPV1 receptor blockade prevents priming of the myocardium for I/R injury following instillation of DEP
ii. $\beta_1$ adrenoceptor and TRPV1 receptor blockade reduces the susceptibility to *ex vivo* I/R injury of hearts from DEP-instilled animals
iii. $\beta_1$ adrenoceptor and TRPV1 receptor blockade reduces DEP-induced pulmonary inflammation

## 6.2 Methods

### 6.2.1 Pulmonary instillation of DEP

Adult male Wistar rats (250-350 g; Charles River Laboratories) were used for all experiments. Rats were instilled intra-tracheally with either DEP (0.5 mg in 0.5 ml saline) or vehicle (saline 0.5 ml). For details of the preparation and instillation process please refer to Chapters 2.2 and 2.3. Animals were sacrificed 6 hours after instillation by exsanguination. This time point was chosen based on results described in Chapter 4.

### 6.2.2 Coronary artery ligation

Ischaemia and reperfusion were performed on rat hearts *ex vivo*. For details of the animal model of myocardial I/R injury please refer to Chapter 2.3.2.

### 6.2.3 Drug treatment studies

For interventional studies with a $\beta_1$ adrenoceptor antagonist rats were randomly assigned to receive either metoprolol (Metoprolol tartate salt, Sigma-Aldrich, UK; 10 mg/kg intraperitoneal (i.p.).; Beique *et al*., 2000; Beril Gok *et al*., 2007) or vehicle (saline) immediately before instillation of DEP or saline. 6 hours later hearts were isolated for ischaemia and reperfusion as described in Chapter 2.3.2. In an additional group, hearts from DEP-instilled animals underwent *ex vivo* I/R while being perfused with Tyrode's buffer containing metoprolol (10 μM; Kovacs *et al*., 2009), to control for any direct *ex vivo* influence of the drug on injury. For interventional studies with a TRPV1 antagonist rats were randomly assigned to receive AMG 9810 ((2E)-N-(2, 3- Dihydro-1, 4-benzodioxin-6-yl)-3-[4-(1, 1- dimethyl)phenyl]-2-propenamide, Tocris, Bioscience, UK; 30 mg/kg intra-tracheal; Gavva *et al*., 2005) dispersed in DEP or saline. 6 hours later hearts were isolated for ischaemia and reperfusion as described in Chapter 2.3.2. In an additional group, hearts from DEP- or saline-instilled animals underwent *ex vivo* I/R while being perfused with Tyrode's

containing AMG 9810 (1 µM), to control for any direct *ex vivo* influence of the drug on injury.

### 6.2.4 Detection of reactive oxygen species (ROS by electron paramagnetic resonance (EPR) spectroscopy

In some *ex vivo* perfused hearts oxygen free radical generation from the myocardium was assessed in perfusion fluid using spin label EPR. Measurements were taken at baseline, after 0 and 30 minutes of ischaemia and after 0, 1 and 2 hours of reperfusion. Please refer to Chapter 2.11 for more details.

### 6.2.5 Tissue collection

For infarct size assessment the whole heart was excised and washed in saline to remove excess Evans Blue prior to storage at -20°C. Wet weight was recorded in some *ex vivo* perfused hearts following reperfusion.

### 6.2.6 Assessment of pulmonary inflammation

In a separate group of animals, a bronchoalveolar lavage (BAL) was performed (as described in Chapter 2.8) 6 hours after 0.5 ml saline or 0.5 mg DEP instillation alone and after 30 mg/kg AMG 9810 or 10 mg/kg metoprolol. Total and differential cell counts were performed on the BAL fluid. Total protein was detected using a bovine serum albumin (BCA) kit (Thermo Scientific Pierce). For details of all the procedures please refer to Chapter 2.8.

### 6.2.7 2, 3, 5-triphenyltetrazolium chloride (TTC) staining

TTC staining was used to assess myocardial viability as described in Chapter 2.6.1. Infarct size was expressed as percentage of the area at risk (AAR).

### 6.2.8 Statistics

All values are expressed as mean ± SEM. Relative radical concentrations (in arbitrary units, AU) were recorded as the signal intensity obtained from the peak-to-

peak height of the first derivative EPR spectrum. In drug intervention studies with AMG 9810, differences in pulmonary inflammatory markers and cardiac viability (before I/R) between the groups were compared by one-way ANOVA. Myocardial AAR, infarct size, oxygen free radical generation and heart weight were analysed by two-way ANOVA. In drug intervention studies with metoprolol, myocardial AAR, infarct size, oxygen free radical generation and heart weight were compared by one-way ANOVA. Pulmonary inflammatory markers and cardiac viability (before I/R) was assessed between groups by a two-way ANOVA. The Bonferroni test was used for post hoc comparisons. $P<0.05$ was considered to be statistically significant.

## 6.3 Results

### 6.3.1 Effect of the $\beta_1$ adrenoceptor antagonist, metoprolol, on cardiac oxidative stress, wet weight and viability prior to I/R

#### 6.3.1.1 Effect of the $\beta_1$ adrenoceptor antagonist, metoprolol, on the DEP-induced cardiac oxidative stress

Baseline oxidant stress, determined by EPR in the perfusate of hearts isolated 6 hours after instillation of saline was 2140.0 ± 224.0 AU (Figure 6.1). Free radical intensity was significantly higher in hearts from DEP-instilled rats ($P<0.01$ compared to saline; Figure 6.1). Administration of metoprolol (10 mg/kg i.p.) at the time of instillation i*n vivo* significantly reduced the magnitude of the EPR signal of hearts from DEP-instilled rats ($P<0.01$ compared to DEP; Figure 6.1).

#### 6.3.1.2 Effect of the β1 adrenoceptor antagonist, metoprolol, on heart wet weight

The wet weight of hearts did not differ between groups (Figure 6.2).

#### 6.3.1.3 Effect of the $\beta_1$ adrenoceptor antagonist, metoprolol, on cardiac myocyte viability

98.4 ± 0.8% of the left ventricle remained viable, as determined by TTC staining, after saline instillation. In agreement with earlier results (see Chapter 5), cell viability was significantly reduced in hearts from DEP-instilled compared to saline-instilled rats ($P<0.001$; Figure 6.3). *In vivo* metoprolol delivery had no effect on cardiac myocyte viability in hearts from saline-instilled rats (Figure 6.3). However, hearts from DEP-instilled rats that received metoprolol (10 mg/kg i.p.) at the time of instillation *in vivo* did not exhibit the loss in cell viability ($P<0.01$; Figure 6.3).

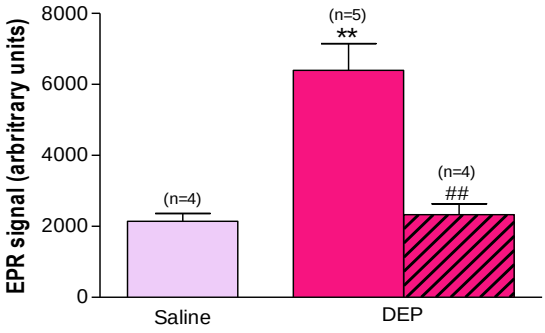


**Figure 6.1 $\beta_1$ adrenoceptor blockade *in vivo* prevented the diesel exhaust particulate (DEP)-induced increase in cardiac oxidative stress.**

Electron paramagnetic resonance (EPR) signal of the 1-hydroxy-3-carboxy-pyrolidine (CP-H) spin adduct at baseline in hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns) or in hearts from DEP-instilled rats that received metoprolol at the time of instillation *in vivo* (10 mg/kg, intraperitoneal, hatched columns). Columns represent mean ± SEM. Numbers in brackets represent n for each group. **P<0.01 versus saline; ##P<0.01 versus DEP; one-way ANOVA followed by Bonferroni post-hoc test.

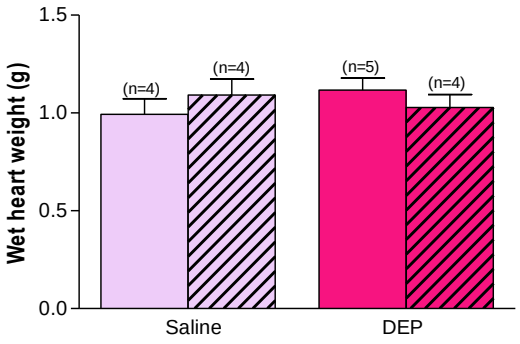


**Figure 6.2 $\beta_1$ adrenoceptor blockade had no effect on wet heart weight.**

Wet weight of hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns) or in hearts from rats that received metoprolol at the time of instillation *in vivo* (10 mg/kg, intraperitoneal, hatched columns). Columns represent mean ± SEM. Numbers in brackets represent n for each group.

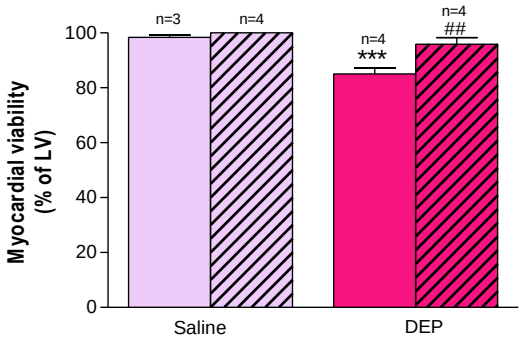


**Figure 6.3 $\beta_1$ adrenoceptor blockade *in vivo* prevented the diesel exhaust particulate (DEP)-induced decrease in cardiac cell viability.**

The percentage of viable myocardium in the left ventricle in hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns) or in hearts from rats that received metoprolol at the time of instillation *in vivo* (10 mg/kg, intraperitoneal, hatched columns) was calculated by deducting the percentage of tissue stained positive with 2, 3, 5-tetrazolium chloride (TTC). Columns represent mean ± SEM. Numbers in brackets represent n for each group. *** P<0.001 versus saline; ##P<0.01 versus DEP; two-way ANOVA with Bonferroni post-hoc test.

## 6.3.2 I/R *ex vivo*

### 6.3.2.1 Effect of the β1 adrenoceptor antagonist, metoprolol, on the increased susceptibility to *ex vivo* I/R injury of hearts from DEP-instilled animals

There were no significant differences between the groups in baseline coronary perfusion pressure (CPP) (Table 6.1)

The AAR was not different between groups (Figure 6.4a). Infarct size in saline-instilled rats averaged 14.7 ± 2.6 % of AAR (Figure 6.4b). In agreement with earlier results (see Chapter 5), infarct size was larger in hearts from DEP-instilled compared to saline-instilled rats ($P<0.001$; Figure 6.4b). Administration of metoprolol (10 mg/kg i.p.) at the time of instillation *in vivo* had no effect on infarct size in hearts from saline-instilled rats (Figure 6.4b). However, hearts from DEP-instilled rats that received metoprolol *in vivo* did not exhibit increased sensitivity to I/R injury ($P<0.001$; Figure 6.4b). Metoprolol had no effect on injury in hearts from DEP-instilled rats when applied only *ex vivo* in the organ bath (Figure 6.4b).

### 6.3.2.2 Effect of the β1 adrenoceptor antagonist, metoprolol, on oxygen free radical production during ischaemia and reperfusion in the isolated perfused rat heart

In all groups the free radical intensities as measured by EPR did not change significantly during any period of ischaemia or reperfusion (Table 6.2).

### 6.3.2.3 Effect of the $\beta_1$ adrenoceptor antagonist, metoprolol, on heart wet weight after *ex vivo* I/R

The wet weight of hearts from rats receiving saline was 1.1 ± 0.1 g (Figure 6.5) and this was significantly increased in hearts from DEP-instilled rats ($P<0.01$; Figure 6.5). *In vivo* metoprolol delivery had no effect on the wet weight of hearts from saline-instilled rats (Figure 6.5). The increased wet weight of hearts from DEP-instilled rats was reversed when metoprolol was administered at the time of DEP instillation *in vivo* and when applied only *ex vivo* in the organ bath (Figure 6.5).

| | Coronary perfusion pressure (CPP) values (mmHg) | | | | |
|---|---|---|---|---|---|
| | Baseline | Onset Isc | 30 min Isc | 1 h Rep | 2 h Rep |
| **Saline** | | | | | |
| Control (n=4) | 52.7 ± 5.4 | 58.4 ± 4.9 | 63.1 ± 15.3 | 60.0 ± 14.4 | 62.7 ± 14.8 |
| 10 mg/kg metoprolol (intraperitoneal) (n=5) | 51.8 ± 6.5 | 53.1 ± 5.4 | 54.2 ± 5.0 | 49.0 ± 4.1 | 52.0 ± 4.0 |
| **DEP** | | | | | |
| Control (n=6) | 52.5 ± 3.0 | 56.6 ± 6.8 | 63.7 ± 9.7 | 58.1 ± 9.6 | 51.8 ± 3.0 |
| 10 mg/kg metoprolol (intraperitoneal) (n=5) | 59.8 ± 2.6 | 63.0 ± 3.9 | 55.5 ± 5.7 | 58.7 ± 4.1 | 56.9 ± 4.0 |
| 10 µM metoprolol (*ex vivo*) (n=4) | 60.5 ± 3.4 | 65.5 ± 8.9 | 64.3 ± 9.8 | 53.3 ± 5.7 | 57.5 ± 6.7 |

**Table 6.1 Coronary perfusion pressures (CPP) sampled at various time intervals during ischaemia and reperfusion from hearts isolated 6 hours after instillation with saline or DEP alone and after 10 mg/kg (intraperitoneal) or 10 µM metoprolol (*in vitro*).**

Abbreviations: DEP = diesel exhaust particulate, Isc = ischaemia, Rep = reperfusion.

Data are expressed as mean ± SEM. Numbers in brackets represent n for each group.

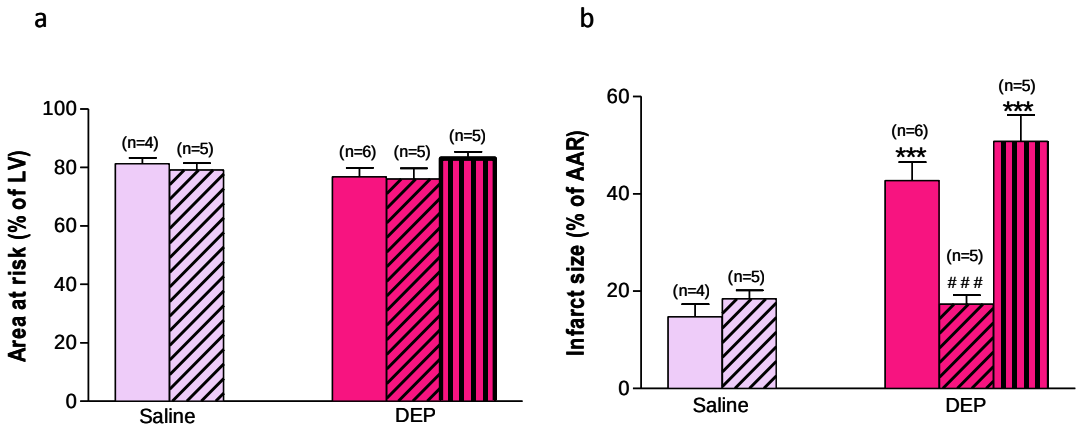


**Figure 6.4 $\beta_1$ adrenoceptor blockade *in vivo*, but not *in vitro*, prevented the diesel exhaust particulate (DEP)-induced increase in infarct size.**

(a) Myocardial area at risk (AAR; expressed as a percentage of total left ventricular (LV) mass) and (b) infarct size (expressed as a percentage of AAR) in hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns); in hearts from rats that received metoprolol at the time of instillation *in vivo* (10 mg/kg, intraperitoneal, hatched columns); or in hearts from DEP-instilled rats perfused *ex vivo* with Tyrode's solution containing metoprolol (10 µM, vertical columns). Columns represent mean ± SEM. Numbers in brackets represent n for each group. ***P<0.001 versus saline; ###P<0.001 versus DEP; one-way ANOVA followed by Bonferroni post-hoc test.

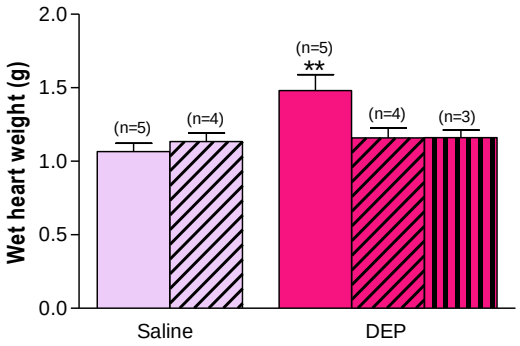


**Figure 6.5 $\beta_1$ adrenoceptor blockade *in vivo* prevented the diesel exhaust particulate (DEP)-induced increase in wet heart weight.**

Wet weight of hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns); in hearts from rats that received metoprolol at the time of instillation *in vivo* (10 mg/kg, intraperitoneal, hatched columns); or in hearts from DEP-instilled rats perfused *ex vivo* with Tyrode's solution containing metoprolol (10 µM, vertical columns). Numbers in brackets represent n for each group. **P<0.01 versus saline; one-way ANOVA followed by Bonferroni post-hoc test.

| | Electron paramagnetic resonance (EPR) signal (arbitrary units) | | | | | |
|---|---|---|---|---|---|---|
| | Baseline | Onset Isc | 30 min Isc | Onset Rep | 1 h Rep | 2 h Rep |
| **Saline** | | | | | | |
| Control (n=4) | 2140.0 ± 224.7 | 2322.3 ± 812.8 | 5342.0 ± 1712.9 | 7714.0 ± 2048.0 | 4366.0 ± 1420.3 | 4279.0 ± 1996.8 |
| **DEP** | | | | | | |
| Control (n=5) | 6393.6 ± 755.2 | 6196.8 ± 499.4 | 7857.6 ± 1564.5 | 9264.0 ± 1123.7 | 6811.2 ± 2059.0 | 4944.0 ± 1062.8 |
| 10 mg/kg metoprolol (intraperitoneal) (n=4) | 2690.7 ± 43.9 | 2621.3 ± 23.2 | 4392.0 ± 120.0 | 5701.3 ± 1820.2 | 4728.0 ± 432.0 | 4744.0 ± 1168.0 |

**Table 6.2 Electron paramagnetic resonance (EPR) intensity in samples taken at different time points during ischaemia and reperfusion from hearts isolated 6 hours after instillation with saline or DEP alone and after 10 mg/kg (intraperitoneal) or 10 µM metoprolol (*ex vivo*).**

Abbreviations: DEP = diesel exhaust particulate, Isc = ischaemia, Rep = reperfusion.

Data are expressed as mean ± SEM. Numbers in brackets represent n per group.

### 6.3.3 Effect of the $\beta_1$ adrenoceptor antagonist, metoprolol, on the DEP-induced pulmonary inflammation

The values for total and differential cell counts in BAL are shown in Figure 6.6. The total number of cells recovered in BAL fluid from saline-instilled rats was 11.0 ± 1.5 x $10^5$ cells/ml (Figure 6.6a). The total number of cells was increased markedly to 77.7 ± 9.5 x $10^5$ cells/ml 6 hours after instillation of DEP ($P<0.001$ compared to saline; Figure 6.6a). Administration of metoprolol (10 mg/kg i.p.) at the time of instillation had no effect on the BAL total cell count of saline-instilled rats (Figure 6.6a), but reduced the total number of cells recovered in BAL fluid from DEP-instilled rats ($P<0.01$ compared to DEP; Figure 6.6a). Differential analysis of BAL cells showed that the BAL from saline-instilled animals was comprised predominately of macrophages (Figure 6.6b). *In vivo* metoprolol delivery had no effect on the macrophage number in the BAL fluid recovered from saline-instilled rats (Figure 6.6b), but significantly increased the number of macrophages recovered in BAL fluid from DEP-instilled rats ($P<0.001$; Figure 6.6b). The increase in BAL total cell count 6 hours after instillation of DEP appeared to be attributed to increases in neutrophil numbers ($P<0.001$ compared to saline; Figure 6.6c). *In vivo* metoprolol delivery had no effect on the neutrophil number in the BAL fluid recovered from saline-instilled rats (Figure 6.6c), but significantly reduced the number of neutrophils recovered in BAL fluid from DEP-instilled rats ($P<0.001$; Figure 6.6c).

Total protein in BAL fluid 6 hours after instillation of DEP was significantly higher than saline-instilled controls ($P<0.01$. Figure 6.6d). *In vivo* metoprolol delivery had no effect on the protein levels in BAL fluid recovered from saline- or DEP-instilled rats (Figure 6.6d).

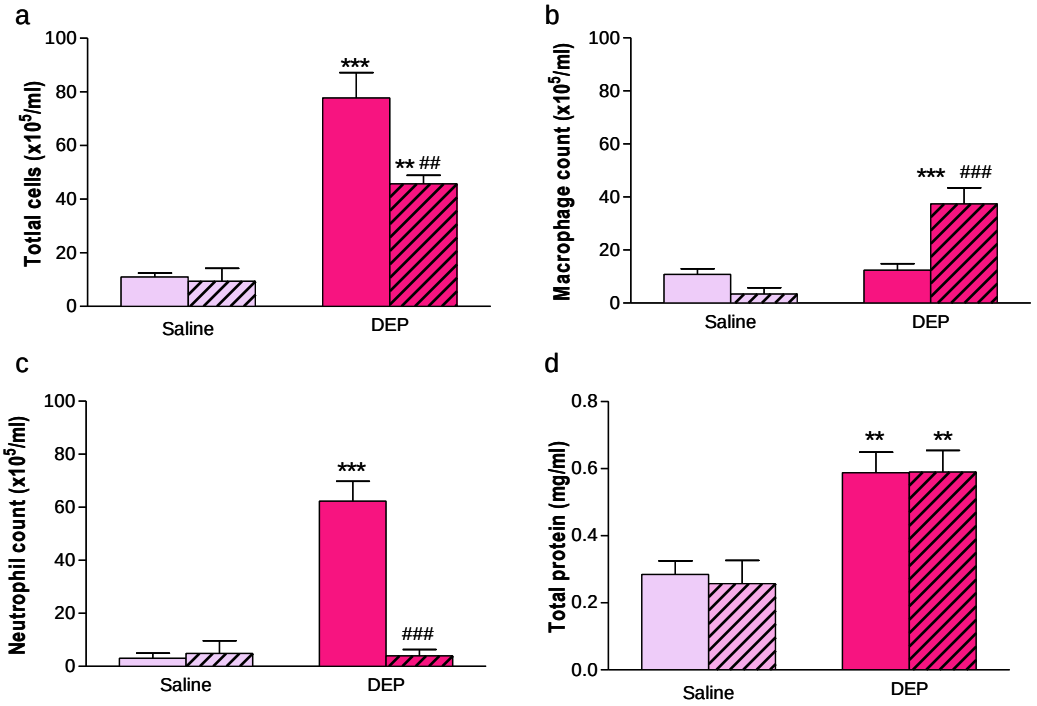


**Figure 6.6 $\beta_1$ adrenoceptor blockade altered the inflammatory cell profile of lungs from rats 6 hours after instillation of diesel exhaust particulate (DEP).**

Bronchoalveolar lavage (BAL) fluid was analysed for (a) total cell count, (b) macrophages, (c) neutrophils and (d) total protein in hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns) or in hearts from rats that received metoprolol at the time of instillation *in vivo* (10 mg/kg, intraperitoneal, hatched columns). Columns represent mean ± SEM (n = 4) **P<0.01, ***P<0.001 versus saline; ##P<0.01, ###P<0.001 versus DEP; two-way ANOVA followed by Bonferroni post-hoc test.

### 6.3.4 Effect of the TRPV1 antagonist AMG 9810 on cardiac oxidative stress, wet weight and viability prior to I/R

#### 6.3.4.1 Effect of the TRPV1 antagonist AMG 9810 on the DEP-induced cardiac oxidative stress

Baseline oxidant stress, determined by EPR in the perfusate of hearts isolated 6 hours after instillation of saline was 2140.0 ± 224.0 AU (Figure 6.7). Free radical intensity was approximately 3-fold higher in hearts from DEP-instilled rats (Figure 6.7). However, the difference did not reach statistical significance possibly due to a lack of power. Values reported here agree with those previously reported in Chapter 5.3.10. *In vivo* AMG 9810 delivery did not affect the magnitude of the EPR signal of hearts from saline-instilled rats (Figure 6.7). Free radical generation from DEP-instilled hearts appeared to be increased further when AMG 9810 was co-administered with DEP *in vivo*, but this failed to reach statistical significance (Figure 6.7). AMG 9810 had no effect on the generation of free radicals measured in hearts from saline-instilled rats when applied only *ex vivo* in the organ bath (Figure 6.7). However, hearts from DEP-instilled rats exhibited significantly higher levels of free radical production when AMG 9810 was applied only *ex vivo* in the organ bath ($P<0.05$; Figure 6.7).

#### 6.3.4.2 Effect of the TRPV1 antagonist AMG 9810 on heart wet weight

The wet weight of hearts did not differ between groups (1.0 ± 0.1 g for 6 hour saline, 1.1 ± 0.0 g for 6 hour DEP and 1.1 ± 0.0 g for 6 hour DEP with 30 mg/kg AMG 9810).

#### 6.3.4.3 Effect of the TRPV1 antagonist AMG 9810 on cardiac myocyte viability

As noted in Chapter 5.3.12, instillation of DEP reduced cardiac myocyte viability as assessed by TTC staining. 85.0 ± 2.1 % of the left ventricle was considered viable in hearts from DEP-instilled rats (Figure 6.8). Co-administration of the TRPV1 antagonist AMG 9810 *in vivo* with DEP into the lung was found to prevent the loss in cardiac myocyte viability ($P<0.05$ compared to DEP; Figure 6.8).

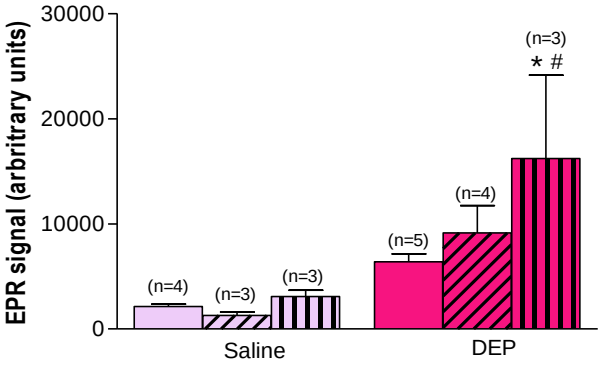


**Figure 6.7 Oxidative stress was increased in hearts from diesel exhaust particulate (DEP)-instilled rats when AMG 9810 was applied only *ex vivo* in the organ bath.**

Electron paramagnetic resonance (EPR) signal of the CPH spin adduct at baseline in hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns); in hearts from rats that received AMG 9810 at the time of instillation *in vivo* (30 mg/kg, intra-tracheal, hatched columns); or in hearts from instilled rats perfused *in vitro* with Tyrode's solution containing AMG 9810 (1 µM, vertical columns). Columns represent mean ± SEM. Numbers in brackets represent n for each group. *P<0.05 versus saline; #P<0.05 versus DEP; two-way ANOVA followed by Bonferroni post-hoc test.

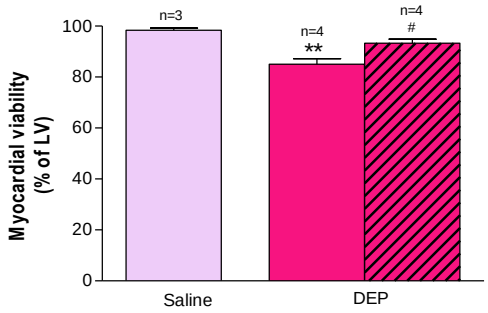


**Figure 6.8 Instillation of a transient receptor potential vanilloid 1 (TRPV1) antagonist with diesel exhaust particulate (DEP) prevented DEP-induced decrease in cardiac cell viability.**

The percentage of viable myocardium in the left ventricle (LV) 6 hours after instillation with 0.5 ml saline (purple columns) or 0.5 mg DEP alone (pink columns) or in hearts from rats that received AMG 9810 at the time of DEP instillation *in vivo* (30 mg/kg, intra-tracheal, pink hatched columns) was calculated by deducting the percentage of tissue stained brick red with 2, 3, 5-tetrazolium chloride (TTC). Columns represent mean ± SEM. Numbers in brackets represent n for each group. ** $P<0.01$ versus saline; #$P<0.05$ versus DEP; one-way ANOVA with Bonferroni post-hoc test.

### 6.3.5 I/R *ex vivo*

#### 6.3.5.1 Effect of the TRPV1 antagonist AMG 9810 on the increased susceptibility to *ex vivo* I/R injury of hearts from DEP-instilled animals

There were no significant differences between the groups in baseline CPP (Table 6.3). There was a tendency towards increased perfusion pressure during the ischaemia and reperfusion, but the increase was not significant (Table 6.3).

The AAR was not different between groups (Figure 6.9a). Infarct size in saline-instilled rats averaged 14.6 ± 2.8 % of AAR (Figure 6.9b). In agreement with earlier results (see Chapter 5), infarct size was larger in hearts from DEP-instilled compared to saline-instilled rats ($P<0.001$; Figure 6.9b). *In vivo* AMG 9810 delivery had no effect on infarct size in hearts from saline-instilled rats (Figure 6.9b). However, hearts from DEP-instilled rats that received AMG 9810 *in vivo* did not exhibit increased sensitivity to I/R injury ($P<0.001$; Figure 6.9b). AMG 9810 had no effect on injury in hearts from saline- or DEP-instilled rats when applied only *ex vivo* in the organ bath (Figure 6.9b).

#### 6.3.5.2 Effect of the TRPV1 antagonist AMG 9810 on oxygen free radical production during ischaemia and reperfusion in the isolated perfused rat heart

In all groups the free radical intensities measured by EPR did not change significantly during any period of ischaemia or reperfusion (Table 6.4). Nevertheless, the observed EPR data did show relatively large variation between- and within-groups. This may have been due to the EPR not being sensitive enough to detect differences. Alternatively, the current study may have been underpowered to identify a significant difference.

#### 6.3.5.3 Effect of the TRPV1 antagonist AMG 9810 on wet weight after *ex vivo* I/R

The wet weight of hearts from rats receiving saline was 1.1 ± 0.1 g (Figure 6.10) and this was significantly increased in hearts from DEP-instilled rats ($P<0.01$; Figure

6.10). *In vivo* AMG 9810 delivery increased the wet weight of hearts from saline-instilled rats but the increase did not reach statistical significance, perhaps due to the small numbers (Figure 6.10). AMG 9810 had no effect on the wet weight of hearts from saline-instilled rats when applied only *ex vivo* in the organ bath (Figure 6.10). The increased wet weight of hearts from DEP-instilled rats was reversed when AMG 9810 was co-administered with DEP *in vivo* and when applied only *ex vivo* in the organ bath (Figure 6.10).

| | Coronary perfusion pressure (CPP) values (mmHg) | | | | |
|---|---|---|---|---|---|
| | Baseline | Onset Isc | 30 min Isc | 1 h Rep | 2 h Rep |
| **Saline** | | | | | |
| Control (n=4) | 49.1 ± 9.3 | 62.8 ± 9.2 | 55.9 ± 5.1 | 50.8 ± 14.9 | 48.2 ± 11.0 |
| 30 mg/kg AMG 9810 (intratracheal) (n=3) | 39.7 ± 3.9 | 47.5 ± 1.3 | 35.3 ± 3.5 | 39.7 ± 4.7 | 45.3 ± 6.4 |
| 1 µM AMG 9810 (*ex vivo*) (n=3) | 45.4 ± 9.0 | 52.8 ± 8.1 | 36.3 ± 7.7 | 41.5 ± 3.8 | 37.6 ± 3.1 |
| **DEP** | | | | | |
| Control (n=5) | 41.6 ± 5.7 | 49.2 ± 5.4 | 40.8 ± 7.2 | 41.1 ± 4.0 | 40.2 ± 6.6 |
| 30 mg/kg AMG 9810 (intratracheal) (n=5) | 55.4 ± 2.2 | 64.1 ± 0.1 | 55.6 ± 2.4 | 54.9 ± 5.8 | 50.8 ± 7.5 |
| 1 µM AMG 9810 (*ex vivo*) (n=3) | 45.2 ± 8.2 | 60.0 ± 9.8 | 59.2 ± 9.8 | 46.8 ± 12.5 | 57.0 ± 3.8 |

**Table 6.3 Coronary perfusion pressures sampled at various time intervals during ischaemia and reperfusion from hearts isolated 6 hours after instillation with saline or DEP alone and after 30 mg/kg (intra-tracheal) or 1 µM AMG 9810 (*in vitro*).**

Abbreviations: DEP = diesel exhaust particulate, Isc = ischaemia, Rep = reperfusion.

Data are expressed as mean ± SEM. Numbers in brackets represent n for each group.

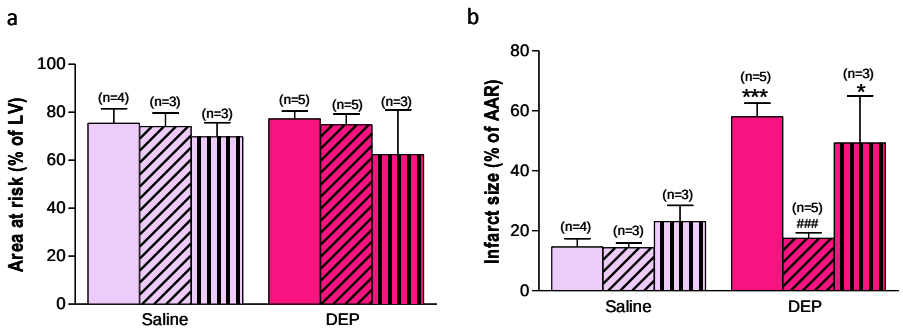


**Figure 6.9 Instillation of a transient receptor potential vanilloid 1 (TRPV1) antagonist with diesel exhaust particulate (DEP) *in vivo*, but not incubation *in vitro*, prevented the DEP-induced increase in infarct size.**

**(a)** Myocardial area at risk (AAR; expressed as a percentage of total left ventricular (LV) mass) and **(b)** infarct size (expressed as a percentage of AAR) in hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns); in hearts from rats that received AMG 9810 at the time of instillation *in vivo* (30 mg/kg, intra-tracheal, hatched columns); or in hearts from instilled rats perfused *in vitro* with Tyrode's solution containing AMG 9810 (1 µM, vertical columns). Columns represent mean ± SEM. Numbers in brackets represent n for each group. *P<0.05, ***P<0.001 versus saline; ###P<0.001 versus DEP; two-way ANOVA followed by Bonferroni post-hoc test.

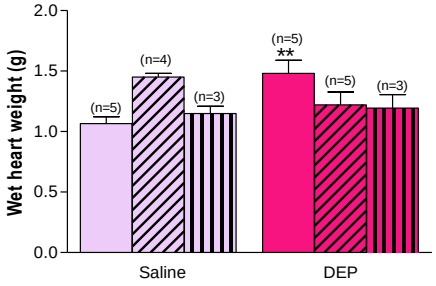


**Figure 6.10 Instillation of a transient receptor potential vanilloid 1 (TRPV1) antagonist with diesel exhaust particulate (DEP) *in vivo* prevented the DEP-induced increase in wet heart weight.**

Wet weight in hearts isolated 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns); in hearts from rats that received AMG 9810 at the time of instillation *in vivo* (30 mg/kg, intra-tracheal, hatched columns); or in hearts from instilled rats perfused *in vitro* with Tyrode's solution containing AMG 9810 (1 µM, vertical columns). Columns represent mean ± SEM. Numbers in brackets represent n for each group. **P<0.01 versus saline; two-way ANOVA followed by Bonferroni post-hoc test.

| | Electron paramagnetic resonance (EPR) signal (arbitrary units) | | | | | |
|---|---|---|---|---|---|---|
| | Baseline | Onset Isc | 30 min Isc | Onset Rep | 1 h Rep | 2 h Rep |
| **Saline** | | | | | | |
| Control (n=4) | 2140.0 ± 224.7 | 2322.3 ± 812.8 | 5342.0 ± 1712.9 | 7714.0 ± 2048.0 | 4366.0 ± 1420.3 | 4279.0 ± 1996.8 |
| 30 mg/kg AMG 9810 (intra-tracheal) (n=3) | 1280.0 ± 341.8 | 1656.0 ± 360.0 | 3528.0 ± 312.0 | 3952.0 ± 395.9 | 8016.0 ± 840.0 | 3032.0 ± 529.5 |
| 1 µM AMG 9810 (*ex vivo*) (n=3) | 3088.0 ± 604.4 | 4902.0 ± 476.2 | 11304.0 ± 5604.3 | 13764.0 ± 6619.3 | 13782.0 ± 7346.0 | 7116.0 ± 5509.4 |
| **DEP** | | | | | | |
| Control (n=5) | 6393.6 ± 755.2 | 6196.8 ± 499.4 | 7857.6 ± 1564.5 | 9264.0 ± 1123.7 | 6811.2 ± 2059.0 | 4944.0 ± 1062.8 |
| 30 mg/kg AMG 9810 (intratracheal) (n=4) | 9144.0 ± 2589.6 | 13881.0 ± 3325.0 | 10545.0 ± 3417.1 | 20169.0 ± 6681.0 | 16011.0 ± 6270.3 | 7516.0 ± 3627.4 |
| 1 µM AMG 9810 (*ex vivo*) (n=3) | 16208.0 ± 7928.1 | 15432.0 ± 7363.2 | 14824.0 ± 7412.5 | 18640.0 ± 7872.9 | 7544.0 ± 3457.1 | 3520.0 ± 2277.5 |

**Table 6.4 Electron paramagnetic resonance (EPR) intensity in samples taken at different time points during ischaemia and reperfusion from hearts isolated 6 hours after instillation with saline or DEP alone and after 30 mg/kg (intra-tracheal) or 1 µM AMG 9810 (*in vitro*).**

Abbreviations: DEP = diesel exhaust particulate, Isc = ischaemia, Rep = reperfusion.

Data are expressed as mean ± SEM. Numbers in brackets represent n for each group

### 6.3.6 Effect of the TRPV1 antagonist AMG 9810 on DEP-induced pulmonary inflammation

The values for total and differential cell counts in BAL are shown in Figure 6.11. The total number of cells recovered in BAL fluid from saline-instilled rats was 11.0 ± 1.5 x $10^5$ cells/ml (Figure 6.11a). The total number of cells was increased markedly to 77.7 ± 9.5 x $10^5$ cells/ml 6 hours after instillation of DEP ($P<0.05$ compared to saline; Figure 6.11a). *In vivo* AMG 9810 delivery caused a small, non-significant decrease in the total number of cells in BAL from DEP-instilled rats (Figure 6.11a). Differential analysis of BAL cells showed that the BAL fluid recovered from saline-instilled rats was comprised predominately of macrophages (Figure 6.11b). The increase in BAL total cell count 6 hours after instillation of DEP appeared to be attributed to increases in neutrophil numbers; rising from 3.1 ± 1.9 x $10^5$ cells/ml in the saline-instilled rats to 62.3 ± 7.5 x $10^5$ cells/ml in DEP-instilled rats ($P<0.05$; Figure 6.11c). *In vivo* AMG 9810 delivery had no effect on the neutrophil number in BAL fluid recovered from DEP-instilled rats (Figure 6.11c).

Total protein in BAL fluid 6 hours after instillation of DEP was significantly higher than saline-instilled controls ($P<0.01$. Figure 6.11d). *In vivo* AMG 9810 delivery caused a small, non-significant decrease on the protein levels in BAL fluid recovered from DEP-instilled rats (Figure 6.11d).

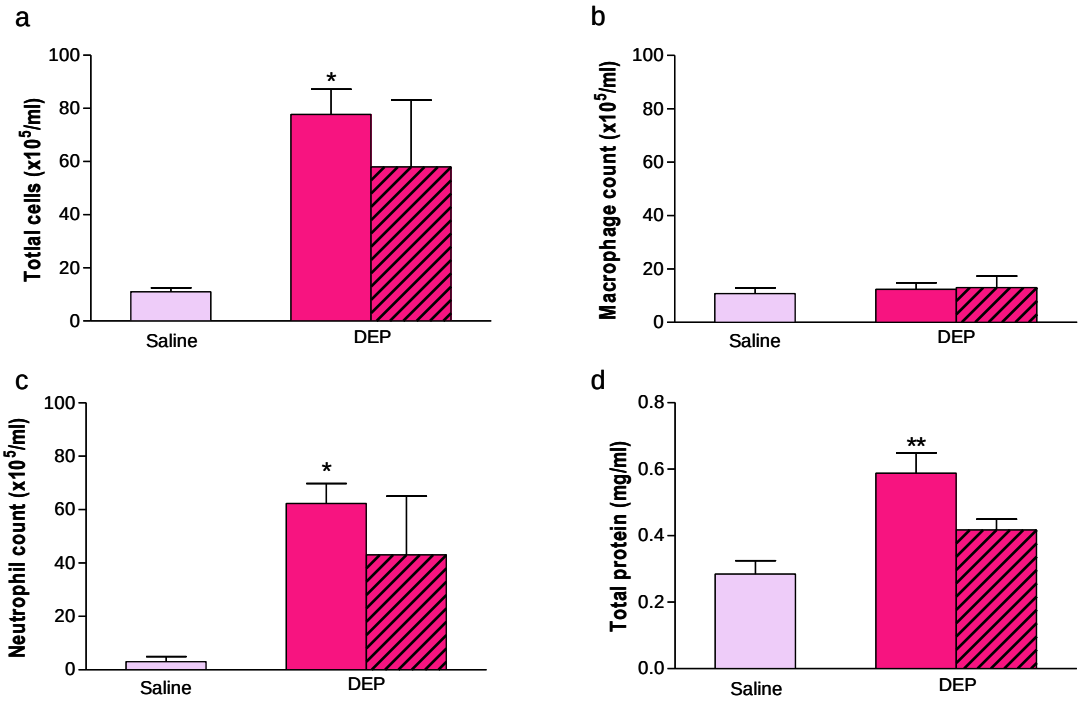


**Figure 6.11 Instillation of a transient receptor potential vanilloid 1 (TRPV1) antagonist with diesel exhaust particulate (DEP) had no effect on DEP-induced pulmonary inflammation.**

Bronchoalveolar lavage (BAL) fluid was analysed for **(a)** total cell count, **(b)** macrophages, **(c)** neutrophils and **(d)** total protein 6 hours after instillation with 0.5 ml saline or 0.5 mg DEP alone (open columns) or when AMG 9810 was co-administered with DEP *in vivo* (30 mg/kg, intra-tracheal, hatched columns). Columns represent mean ± SEM (n = 4) *P<0.05, compared to saline; one-way ANOVA with Bonferroni post-hoc test.

## 6.4 Discussion

In Chapter 5 it was demonstrated that a single exposure of the lung to DEP leads to priming of the myocardium for increased I/R injury. In the current study, this heightened sensitivity to reperfusion injury was shown to be abrogated both when the $\beta_1$ adrenoceptor antagonist, metoprolol, and when the TRPV1 antagonist, AMG 9810 were administered *in vivo* at the time of instillation. Together, these results suggest the possibility that a TRPV1-dependent neuronal reflex response triggered by DEP deposition in the lung contributes to the increased sensitivity to I/R injury by causing secondary activation of systemic $\beta_1$ adrenoceptors.

### 6.4.1 Role of $\beta_1$ adrenoceptors in DEP-induced increased sensitivity to I/R injury

The research described in Chapter 5 demonstrated that the ability of the myocardium to withstand ischaemic conditions was additionally compromised when normal healthy rats were exposed, by intra-tracheal instillation, to DEP. Sensitisation of the rat myocardium occurred within 6 hours of exposure, which correlates well with a recent clinical study identifying this as the period of greatest risk for myocardial infarction (MI) associated hospitalisation after exposure to atmospheric pollution (Bhaskaran *et al.*, 2011). Deaths during this period were due to ischaemia-evoked arrhythmia, while ischaemia-induced injury was also increased. Furthermore, experiments performed in isolated perfused rat hearts 6 hours after DEP exposure demonstrated that exposure primed the hearts for subsequent injury and that enhancement of inflammatory cell recruitment was not required. Priming was associated with increased myocardial oxidative stress, accompanied by a loss in cardiomyocyte viability.

Sympathetic drive is a key regulator of cardiac rhythm and decreases in HRV, indicative of increased sympathetic tone, have been reported both in humans and in animals after exposure to various components of air pollution (Brook *et al.*, 2009a; Gold *et al.*, 1999; Pope *et al.*, 1999; Rivero *et al.*, 2005; Schulz *et al.*, 2005).

Catecholamines also have been reported to exert toxic effects on the heart, including oxidative stress and apoptosis (Communal *et al.*, 1998; Izem-Meziane *et al.*, 2012; Romeo *et al.*, 2000), both features of hearts collected after DEP exposure (see Chapter 5). As $\beta_1$ adrenoceptor signalling pathways have been implicated in mediating these effects (Communal *et al.*, 1998; Park *et al.*, 1999; Romeo *et al.*, 2000), the ability of $\beta_1$ adrenoceptor blockade to modify DEP induced injury and sensitisation to reperfusion injury was investigated. In the current study, administration of metoprolol (10 mg/kg i.p.), which targets $\beta_1$ adrenoceptors, at the time of instillation *in vivo* was found to prevent enhancement of *ex vivo* reperfusion injury associated with DEP instillation. This result suggests an autonomic shift towards increased sympathetic drive after exposure to DEP making the heart more susceptible to subsequent myocardial injury. Of note, the raised blood pressure and increased sensitivity to arrhythmias during ischaemia observed in the DEP-instilled animals are supportive of the hypothesised increase in sympathetic activity (see Chapter 5).

The present study, supporting an autonomic component in particle mediated cardiac toxicity is consistent with other experimental studies (Ghelfi *et al.*, 2008; Hazari *et al.*, 2011; Rhoden *et al.*, 2005; Wellenius *et al.*, 2002). Ghelfi *et al*. (2008) reported particulate matter (PM)-induced changes in ventricular depolarisation and repolarisation to be mediated by sympathetic activation. These conduction abnormalities have been implicated in predisposing the heart to drug-induced arrhythmia (Hazari *et al.*, 2009; Hazari *et al.*, 2011). Importantly, exposure to PM alters not only sympathetic but also parasympathetic control of the heart (Ghelfi *et al.*, 2008; Rhoden *et al.*, 2005). For example, Rhoden and colleagues observed co-activation of both autonomic branches following exposure to CAP (Rhoden *et al.*, 2005). More recently, a single inhalation exposure to transition metal-rich PM in rats resulted in a reduced ratio of low-frequency to high-frequency power (measure of HRV), suggesting parasympathetic dominance (Farraj *et al.*, 2011). Future studies should be extended to evaluate whether parasympathetic antagonists also ameliorate the effects of DEP exposure on the heart. Moreover, measurement of HRV will

strengthen understanding of the changes in the regulation of the autonomic nervous system (ANS) elicited by DEP exposure.

The exact mechanism by which the $\beta_1$ adrenoceptor antagonist influences susceptibility to subsequent myocardial injury in the present study is unclear, but may be due to reduced oxidative stress and cell death. This is in line with a previous study that found administration of a sympathetic antagonist to effectively block CAP-induced cardiac oxidative stress (Rhoden *et al.*, 2005). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase distributed within heart cells (Cave *et al.*, 2005) was discussed in Chapter 5 as a potential intracellular source of the increased ROS production in DEP-instilled rats. *In vitro* studies have shown that alpha 1 ($\alpha_1$) adrenoceptor stimulation induces ROS in adult rat ventricular myocytes, at least partially, through the activation of NADPH oxidase (Shattock *et al.*, 1991). Future studies using inhibitors of the NADPH oxidase complex could be useful in determining a possible linkage of $\beta_1$ adrenoceptor stimulation to cardiomyocyte apoptosis through NADPH oxidase. It cannot, however, be entirely ruled out that the increased myocardial viability, as assessed by TTC, and the reduced number of apoptotic cells (see Appendix 5), were the result of metoprolol's anti-inflammatory effects on the tissue. *In vitro* studies have demonstrated that tumour necrosis factor alpha (TNFα) induces apoptosis in cardiac myocytes (Dabisch *et al.*, 2004). Metoprolol has been shown to reduce myocardial expression of this and other inflammatory cytokines including, interleukin-6 (IL-6) and interleukin-1 beta (IL1β) in rat models of both microembolisation (Lu *et al.*, 2011) and coronary artery ligation (Cheng *et al.*, 2005). However, as reported in Chapter 5 there were no infiltrating neutrophils detectable on haematoxylin and eosin (H & E) stained paraffin sections from hearts of animals instilled with DEP 6 hours previously, suggesting that anti-inflammatory effects of metoprolol are unlikely to be of major importance. Although not studied here, a decrease in blood pressure may provide another possible explanation for the decreased I/R sensitivity in DEP-instilled rats that received metoprolol *in vivo*. In previous studies in rats, short-term administration of metoprolol to spontaneously hypertensive (SH) rats resulted in decreases in arterial

blood pressure (Åblad et al., 1984). Determining the *in vivo* effects of metoprolol on blood pressure clearly requires further investigation.

A note of caution must be exercised in interpreting the findings from the current study. It is unclear whether DEP targets the NTS, which regulates outgoing autonomic signals to the heart, through afferent fibres of the vagus nerve (discussed in more detail below) or indirectly via the circulation. As discussed below DEP-induced pulmonary inflammation was not completely abrogated by administration of metoprolol *in vivo* at the time of instillation. Moreover, no up-regulation of neutrophil adhesion or circulating pro-inflammatory cytokines was detected after DEP exposure in the studies outlined in Chapters 4 and 5. Collectively these results point towards autonomic imbalance and increased sensitivity to I/R injury by DEP through nerve impulses transmitted from lung to brain rather than indirect effects of inflammation.

Finally, in the present study neither metoprolol nor AMG 9810 (discussed in more detail below) appeared to affect CPP at baseline or during I/R when compared with DEP-instilled animals. These findings suggest that alterations in coronary endothelial cell function (Broadley, 1979) were not a cause of the reduced infarct size demonstrated with the adrenoceptor and TRPV1 antagonists, although further studies would be necessary to confirm this. Additionally, in the present study AAR did not differ between groups, showing that the reduced infarct size was not due to a smaller ischaemic zone.

### 6.4.2 Role of $\beta_1$ adrenoceptors in DEP-induced pulmonary inflammation

Surprisingly, administration of metoprolol (10 mg/kg i.p.) *in vivo* at the time of instillation resulted in a reduction in total cell and neutrophil numbers in BAL fluid recovered from DEP-instilled rats. The reasons for this are unclear but could be, in part, due to the expression of $\beta_1$ adrenoceptors on rat lung (Xue *et al.*, 1983). Landiolol, a short-acting selective $\beta_1$ adrenoceptor-blocking agent has been reported to attenuate acute lung injury in a rat model of endotoxin-induced sepsis (Hagiwara

*et al.*, 2009). The increased lung macrophage number in the present study may be due to recruitment of macrophages from the circulation to engulf apoptotic neutrophils during the resolution of inflammation (Gilroy *et al.*, 2004). It is important to note that not all pulmonary inflammatory effects (BAL total protein) of DEP were abolished by administration of metoprolol *in vivo* at the time of instillation. Thus the cardiovascular changes in response to DEP cannot entirely be attributed to secondary effects of inflammation.

In summary, the heightened sensitivity to *ex vivo* I/R injury in DEP-instilled rats was abrogated when the $\beta_1$ adrenoceptor antagonist, metoprolol was administered *in vivo* at the time of instillation. This antagonist was also effective in blocking the DEP-induced cardiac oxidative stress and cell death. However, further studies are needed to fully establish whether these effects are primarily credited to metoprolol's anti-inflammatory effects on DEP-induced pulmonary inflammation.

### 6.4.3 Role of TRPV1channels in DEP-induced increased sensitivity to I/R injury

The experimental evidence discussed above strongly suggests that activation of the SNS following DEP exposure is central to the priming of the myocardium for increased I/R injury. It has been suggested that lung C-fibre receptor activation modifies sympathetic and parasympathetic output via feedback to the CNS. TRPV1 has been found to be expressed both at mRNA and protein levels in C-fibre neurons innervating the lung and airways (Groneberg *et al.*, 2004; Ni *et al.*, 2006; Seki *et al.*, 2006; Watanabe *et al.*, 2005). A more recent study has shown activation of neuronal TRPV1 receptors *in vitro* by DEP (Deering-Rice *et al.*, 2011). In the current study co-instillation of AMG 9810 with DEP *in vivo* was found to prevent enhancement of *ex vivo* reperfusion injury associated with DEP instillation. These data suggest an initiating role for TRPV1 in mediating the increased sensitivity of hearts to *ex vivo* I/R injury associated with DEP exposure. Prior research has reported that triggering of TRPV1-mediated autonomic reflexes in the lung may, at least in part, explain adverse cardiovascular responses to pollutants (Ghelfi *et al.*, 2008). A direct effect of translocated DEP (or its associated constituents) on TRPV1 receptors in extra-

pulmonary tissues can also not be ruled out. Indeed, the presence of TRPV1 receptors has been reported in tissues other than the lung including, pancreas, liver, and heart (O'Neil *et al.*, 2003; Zahner *et al.*, 2003). However, as discussed in the previous chapter, if DEP did translocate into the systemic circulation it seems unlikely that particles would account for the full biological effects of DEP entering the lung. Of note, there is evidence from pharmacological (Hu *et al.*, 2002; Rang *et al.*, 2004) and gene knockout (KO) studies (Wang *et al.*, 2005) that activation of TRPV1 receptors on the heart can trigger protective mechanisms that may attenuate I/R injury. For example, Wang and colleagues demonstrated TRPV1 gene deletion to impair post-ischaemic recovery in isolated mouse hearts (Wang *et al.*, 2005). Thus, activation of TRPV1 receptors in the heart would have been expected to afford protection from the increased sensitivity to I/R injury by DEP. Moreover, AMG 9810 had no effect on I/R injury in hearts when applied only *ex vivo* in the organ bath. Collectively, these results point towards TRPV1 receptors in the lung, but not in the heart, conveying the increased sensitivity to I/R injury induced by exposure to DEP.

As discussed in Chapter 5, exposure to DEP significantly reduced cardiac cell viability due to apoptotic cell death. The loss of viable cardiac tissue, as assessed by TTC, was prevented by co-instillation of AMG 9810 with DEP *in vivo*. Moreover, inhibition of TRPV1 receptors in the lungs was also shown to prevent apoptotic myocyte loss induced by DEP (see Appendix 5). Previous *in vitro* studies have demonstrated the importance of TRPV1 in mediating PM-induced apoptosis (Agopyan *et al.*, 2004; Agopyan *et al.*, 2003). In human airway epithelial cell lines Agopyan and colleagues reported that the influx of extracellular calcium ($Ca^{2+}$) through TRPV1 causes the PM-induced cell death (Agopyan *et al.*, 2004; Agopyan *et al.*, 2003). However, others have shown removal and chelation of $Ca^{2+}$ to be ineffective in ameliorating cell death (Reilly *et al.*, 2005; Reilly *et al.*, 2003). It is worth noting that in these studies the authors have made the assumption that PM can definitely translocate to extra-pulmonary tissues. However, as discussed in earlier chapters, a potential role of PM translocation remains somewhat controversial.

In the preceding chapter, it was hypothesised that cardiac oxidative stress plays a causal role in the cardiac cell death associated with DEP exposure. It was predicted, therefore, that the increased oxidative stress in hearts would be prevented by blocking TRPV1 receptors in the lungs at the time of DEP exposure. Surprisingly, co-instillation of AMG 9810 with DEP *in vivo* had no influence on the oxidative stress generation. In contrast, pre-treatment with the TRPV1 antagonist capsazepine (CPZ, delivered by aerosol inhalation) was found to prevent enhanced cardiac oxidative stress induced by CAP (Ghelfi *et al.*, 2008). This disparity may be due to use of a different TRPV1 receptor antagonist in the current study from the above-cited study by Ghelfi *et al*. (2008). This is supported by studies reporting CPZ to have low TRPV1 specificity (Behrendt *et al.*, 2004) and poor pharmacokinetic properties (Valenzano *et al.*, 2004). Thus, it is unknown whether the adverse cardiac effects demonstrated in the previous study by CAP are truly mediated by TRPV1 or independent of TRPV1 binding. Compared with CPZ, AMG 9810 used in the current study has been demonstrated to exhibit greater potency, affinity and selectivity for TRPV1 (Gavva *et al.*, 2005).

It is also worthwhile noting that distinct pools of TRPV1 within cells have been linked to different downstream effects (Reilly *et al.*, 2005) and may provide a possible explanation to why the TRPV1 antagonist could not protect the heart from DEP-induced oxidative stress. It is currently unclear whether AMG 9810 blocked all sub-cellular pools of TRPV1. We can also not rule out the possibility that AMG 9810 leaves the lungs and enters the circulation. Blocking TRPV1 receptors in sensory nerve fibres innervating the heart might, in turn, negate possible anti-oxidant effects of blocking its activity in the lung. A possible role of cardiac TRPV1 receptors in protecting the heart from I/R injury was discussed above. At present the mechanisms underlying TRPV1-induced cardiac protection are unclear. There is limited evidence to suggest that such protection is mediated by sensory neuropeptides released on TRPV1 activation (Li *et al.*, 1996; Wang *et al.*, 2005). Intriguingly, NADPH oxidase-derived ROS have been implicated in the protection afforded by these sensory neuropeptides (Starr *et al.*, 2008). Based on this, one would have expected co-instillation of AMG 9810 with DEP *in vivo* to decrease

rather than increase ROS formation in hearts. Of note, addition of AMG 9810 to the heart perfusate *ex vivo* significantly increased ROS generation, suggesting that activation of TRPV1 receptors on the heart do not contribute to the increased ROS generation in hearts of rats exposed to DEP.

To summarise these data strongly imply that DEP-induced cardiac cell death was not secondary to oxidative stress. This is strengthened by other lines of evidence showing anti-oxidants to be ineffective in decreasing the number of dead cells after PM exposure in epithelial cell lines (Li *et al.*, 2002). Additionally, these findings suggest that more than one target may be involved in mediating the heightened myocardial injury associated with DEP. *In vitro* studies have shown that neutral synthetic polymer microsphere (SPM)-mediated IL-6 release was only partially inhibited by pre-treatment of CPZ in dorsal root ganglia (DRG) neurons and epithelial cells (Oortgiesen *et al.*, 2000).

### 6.4.4 Effect of TRPV1 blockade on DEP-induced pulmonary inflammation

So far, this discussion has focussed primarily on the activation of TRPV1 receptors in pulmonary C-fibres transmitting signals to the CNS. However, activation of TRPV1 receptors in pulmonary C-fibre sensory nerves may also trigger the release of neuropeptides (e.g. SP and CGRP) through a local-axon reflex, resulting in an inflammatory response (neurogenic inflammation) (Holzer, 1988). Moreover, TRPV1 receptors localised in non-neuronal cells such as airway epithelial cells and mast cells (Biro *et al.*, 1998; Veronesi *et al.*, 2006) can initiate and enhance the inflammatory response by releasing cytokines. *In vitro* studies have demonstrated TRPV1-mediated pro-inflammatory cytokine production in several human respiratory epithelial cell lines (Reilly *et al.*, 2003; Seki *et al.*, 2007). *In vivo* studies with rats have also reported respiratory inflammation in response to inhalation of the TRPV1 agonist, capsaicin (Reilly *et al.*, 2003). Conflicting results have been reported in studies evaluating a causal relationship between TRP channels and PM-induced airway inflammation (Oortgiesen *et al.*, 2000; Veronesi *et al.*, 1999b; Witten *et al.*, 2005; Wong *et al.*, 2003). In the human epithelial cell line BEAS-2B-

increased generation of IL-6 by ROFA was inhibited by CPZ pre-treatment (Veronesi *et al.*, 1999b). Similar results have been reported on isolated mouse vagal pulmonary sensory neurons (Veronesi *et al.*, 2000). Other studies have, however, concluded that lung C-fibre activation does not play a dominant role in pollution-induced pulmonary inflammation and injury (Wong *et al.*, 2003; Zweier *et al.*, 2006). Studies in Chapter 4 demonstrated pro-inflammatory cytokine concentrations as well as neutrophil counts to be higher in BAL fluid of rats instilled with DEP. Results described in this chapter show that the cardiovascular responses to DEP cannot be entirely attributed to an inflammatory response following TRPV1 activation. While there was a tendency towards reduced lung inflammation, as measured by inflammatory indices in BAL fluid, in DEP-instilled rats that received AMG 9810 *in vivo* this did not reach statistical significance, perhaps due to the relatively small sample size. This result suggests that the adverse cardiovascular responses to DEP are, at least in part, directly related to centrally-mediated reflexes. It will be important, however, for future experiments to determine whether the deleterious effects of DEP are related to endogenous release of SP and CGRP, via activation of TRPV1 receptors, with the use of antagonists of the SP and CGRP receptor.

In summary, co-instillation of AMG 9810 with DEP *in vivo* prevented the heightened sensitivity to *ex vivo* I/R injury associated with DEP instillation. Moreover, studies assessing the pulmonary inflammatory response of AMG 9810 suggest DEP deposition in the lung triggers TRPV1-mediated central reflexes, which in turn renders hearts more sensitive to I/R injury.

### 6.4.5 Future studies and limitations

There are a number of limitations to be noted regarding this study and several questions remain unanswered.

$\beta_1$ adrenoceptor protein expression has been reported in several tissues other than heart, including the liver and kidney (Hellgren *et al.*, 2000), to mediate a diverse array of cellular responses. Therefore, methods such as microneurography, HRV and baroreflex sensitivity assessment should be used to confirm increased sympathetic

activity by pulmonary exposure to DEP. It will also be necessary to investigate the effects of TRPV1 blockade on the ANS function in DEP-instilled rats.

The present study is also limited in that it did not address the potential contribution of other TRP channels in mediating the heightened sensitivity to myocardial injury following exposure to DEP. Activation of TRP ankyrin 1 (TRPA1), in addition to TRPV1, by DEP was recently reported in primary cultures of DRG neurons (Deering-Rice *et al.*, 2011). Other studies have shown DEP to increase activation of TRPV4 in primary human respiratory epithelial cell lines (Deering-Rice *et al.*, 2011). This may help explain the apparent discrepancies between the cytotoxicity and oxidative data, and the inability of AMG 9810 to block oxidative stress. Future studies using genetic ablation of TRP channels and/or pharmacological channel inhibition are therefore warranted.

Experimental studies are also needed to evaluate TRPV1 blockade and $\beta_1$ adrenoeceptor blockade on I/R injury *in vivo* following exposure to DEP. Previous studies reported in Chapter 5 demonstrated the ability of a single exposure to DEP to increase the vulnerability of the heart to developing arrhythmia during *in vivo* ischaemia and reperfusion. Although it was not studied here we predict that TRPV1 mediates the heightened sensitivity to arrhythmia during ischaemia. Pre-treatment with a TRPA1 antagonist prevented the heightened sensitivity to aconitine-induced cardiac arrhythmias reported in rats after exposure to whole diesel exhaust (wDE) (Hazari *et al.*, 2011). A similar effect was observed following sympathetic modulation with guanethidine (Hazari *et al.*, 2011).

Furthermore, the mechanism of activation of TRPV1 by PM is not completely understood. Agopyon *et al*. (2003) and others (Oortgiesen *et al.*, 2000) have demonstrated that the effect of PM on TRPV1 is strongly dependent on PM carrying a net-negative surface charge. Neutral SPM was markedly less effective than its charged counterparts in increasing intracellular $Ca^{2+}$ and IL-6 in the mouse DRG (Oortgiesen *et al.*, 2000). Electrophilic components of DEP have been reported to bind to TRPA1 via the formation of reversible covalent bonds with cysteine residues

present in the ion channel (Deering-Rice *et al.*, 2011). These receptors can also be activated by ROS that are produced in the lung when inflammation accompanies exposure to PM (Bessac *et al.*, 2008). In cultured DRG (Story *et al.*, 2003) and trigeminal ganglia (TG) (Jordt *et al.*, 2004) cells, TRPA1 has been shown to co-localise with TRPV1. Moreover, it has been reported that TRPV1 is activated secondary to TRPA1 perhaps due to $Ca^{2+}$ influx or cytokine production (Skrzypiec-Spring *et al.*, 2007).

Lastly, the effects of DEP instillation on TRPV1 expression were not investigated here. Polymerase chain reaction (PCR) studies have shown that TRPV1 mRNA expression in lungs increases after PM exposure *in vivo* (Costa *et al.*, 2011) and *in vitro* (Veronesi *et al.*, 2003). Moreover, increases in the level of TRPV1 expression has been shown to correlate with increased sensitivity of TRPV1 to noxious stimuli (Reilly *et al.*, 2005). Whether DEP instillation increases the expression of TRPV1 mRNA and receptor protein and potentially decreases tolerance during subsequent exposures warrants investigation.

### 6.4.6 Conclusion

Collectively the data presented here suggest that a TRPV1-dependent neuronal reflex response triggered by DEP deposition in the lung contributes to the increased sensitivity to I/R injury by causing secondary activation of systemic $\beta_1$ adrenoeceptors.

# Chapter 7

# General discussion

Exposure to air pollution has been associated with increased cardiovascular mortality and morbidity (Bhatnagar, 2006; Brook *et al.*, 2003; Dockery *et al.*, 1993). These associations are strongest for the particulate matter (PM) in air pollution (Brook, 2008; Mills *et al.*, 2009). Ultrafine particles (UFP; or nanoparticles) are of specific concern because their small size allows them to penetrate deep into the respiratory tract and also engenders them with a large surface area (Oberdorster, 2001). Exhaust from diesel engines is especially rich in nanoparticles and, therefore, may contribute greatly to the health effects of PM in urban environments (Baulig *et al.*, 2003; Lucking *et al.*, 2011).

There are numerous clinical and experimental observations showing detrimental effects of diesel exhaust (DE) and other PM on coronary vascular function, on atherosclerotic plaque development and stability and on thrombosis and clot resolution (Kunzli *et al.*, 2005; Nemmar *et al.*, 2003a; Suwa *et al.*, 2002). A previous study in this laboratory demonstrated enhancement of myocardial ischaemia in patients with stable coronary heart disease during acute exposure to DE (Mills *et al.*, 2007). While the coronary vascular and pro-thrombotic effects of PM increase the likelihood of heart attack, there is also evidence that exposure to PM has deleterious effects on the myocardium itself. Peters *et al.*, (2000) and others (Dockery *et al.*, 2005; Rich *et al.*, 2005) have reported that exposure to PM increases the incidence of ventricular arrhythmias in patients with implantable defibrillators. Experimental studies have also reported cardiac abnormalities, including impaired left ventricular diastolic performance and arrhythmias after *in vivo* exposures to PM (Anselme *et al.*, 2007; Yan *et al.*, 2008). These observations suggest that pulmonary exposure to PM may also render the heart more vulnerable to injury following infarctions. To date, only one study has addressed this question directly:- myocardial ischaemia/reperfusion (I/R) injury was increased in mice 24 hours after pulmonary instillation of concentrated ambient particles (CAP) (Cozzi *et al.*, 2006). However, the mechanism by which pulmonary exposure to pollution is associated with enhanced reperfusion injury is currently unclear.

The work described in this thesis was designed to investigate the hypothesis that intra-tracheal instillation of diesel exhaust particulate (DEP) in rats would increase the vulnerability of the heart to subsequent I/R injury. Furthermore, markers of inflammation, and of endothelial and autonomic function, were assessed in order to elucidate potential mechanisms of actions. Changes in all these parameters have been proposed as potential mechanisms linking air pollution with adverse cardiovascular events (Brook, 2008; Brook *et al.*, 2004).

## 7.1 Exposure to DEP increases arterial blood pressure

Blood pressure was higher 6 hours, but not 24 hours, after instillation with DEP relative to saline-instilled controls (Chapter 4). The higher blood pressure in the DEP-instilled rats was not associated with a systemic inflammatory response (no change in circulating interleukin-6 (IL-6), C reactive protein (CRP) or tumor necrosis factor alpha (TNFα)). Additionally, alterations in endothelial function and subsequent changes in vasomotor tone are unlikely as acetylcholine (ACh)-induced dilation was not altered in the hind-limb vascular bed (Chapter 4). Based on data generated showing DEP-induced cardiac injury (oxidative stress and cell death) could be prevented by a beta 1 ($\beta_1$) adrenoceptor antagonist (Chapter 6), a more likely explanation is that the increase in blood pressure was due to an increase in sympathetic tone. In support of this, previous animal and human studies have reported PM-induced elevations in blood pressure to be mediated by neurohumoral activation (Bartoli *et al.*, 2009b; Brook *et al.*, 2009a). Bartoli *et al*. (2008) demonstrated that the raised diastolic blood pressure (DBP) during a 5 hour exposure to CAP was significantly attenuated by prazosin, a selective alpha 1 ($\alpha_1$) adrenoceptor antagonist. Thus, studies with α adrenoceptor antagonists are needed to confirm the involvement of the sympathetic nervous system (SNS) in the increase in blood pressure.

## 7.2 DEP exposure does not impair endothelial cell function *ex vivo* or *in vivo*

Experiments performed on the intact hind-limb vasculature *in vivo*, revealed exposure to DEP to have no effect on the vasodilator responses to the endothelium-dependent vasodilator ACh, at 6 or 24 hours post-exposure (Chapter 4). These *in vivo* measurements were supported by a lack of effect of DEP on vascular function *ex vivo* (Appendix 2). In Chapter 4, these results were discussed in detail in relation to other studies using DEP and other PM.

Collateral blood flow is an important determinant of infarct size (Reimer *et al.*, 1981). Endothelial cell dysfunction *in vivo* following DEP instillation may make the heart more prone to reperfusion injury, perhaps by compromising blood flow and enhancing inflammatory cell infiltration. Of note, the present data evaluating endothelial cell function in the rat hind-limb vascular bed cannot be directly extrapolated to the heart. Nevertheless, studies have established a close relation between the endothelial cell function of the peripheral circulation and that of the coronary circulation (Anderson *et al.*, 1995). Therefore, the *in vivo* endothelial cell function data may indicate that impairment of endothelial function in coronary collateral vessels does not play a key role in the increased ischaemic vulnerability of the heart in rats exposed to DEP by instillation (Chapter 5). In further support of this conclusion, endothelial cell dysfunction would have been expected to induce changes in coronary perfusion pressure (CPP). As described in Chapter 5, CPP was not different in hearts isolated from DEP exposed compared to vehicle treated rats.

It is also important to mention that endothelial cell function was preserved at times when there was significant pulmonary (6 hour) and systemic (24 hour) inflammation. It has been reported that inflammation after PM exposure plays a role in mediating cardiovascular responses (Seaton *et al.*, 1995). Consistent with previous work (Madden *et al.*, 2000; Nemmar *et al.*, 2003a), pulmonary inflammation was detected at 6 hours after DEP exposure. In contrast, systemic inflammation was only detectable at 24 hours, when pulmonary inflammation had resolved. The time course of these responses could suggest that these factors originate from the prior

inflammatory response in the lung. Furthermore, these data show that inflammation alone is unlikely to account for the cardiovascular actions of inhaled particulates. This was further supported by the observation that sensitisation to myocardial I/R-induced injury could be reproduced in the isolated Langendorff-perfused rat heart.

Unlike the responses to ACh, the relaxation produced by the endothelium-independent vasodilator sodium nitroprusside (SNP) *in vivo* was impaired both 6 and 24 hours after administration of DEP (Chapter 4). The preserved dilator response to SNP *ex vivo* (Appendix 2) strongly suggests that autonomic alterations likely contribute to the impaired dilator responses observed *in vivo*. Changes in autonomic and baroreceptor activity have been reported following PM exposure (Gold *et al.*, 1999; McQueen *et al.*, 2007; Pope *et al.*, 1999; Rhoden *et al.*, 2005; Wellenius *et al.*, 2002). It is possible, therefore, that the reduced vasodilator response to SNP in the DEP-instilled rats was due to baroreflex-induced vasoconstriction in response to SNP-induced hypotension. The decrease in mean arterial pressure caused by SNP was not different between the saline and DEP group. The latter suggests that exposure to DEP may lead to reduced baroreceptor sensitivity and increased vasoconstriction, thereby opposing the vasodilator effect of SNP. Baroreceptor sensitivity should be assessed in future studies, for example by the assessment of changes in blood pressure against heart rate (HR) induced by intravenous bolus injections of phenylephrine (Chang *et al.*, 1986).

## 7.3 DEP exposure causes myocardial oxidative stress and cell death

As mentioned above, exposure to DEP caused pulmonary inflammation at 6 hours, which had largely resolved by 24 hour post-exposure. This observation, coupled with the previous demonstrations that blood pressure and circulating inflammatory cytokines were also elevated at 6 hours, led to this time-point being selected for subsequent investigations.

Instillation of DEP alone was associated with increased myocardial oxidative stress, as measured by an increase in the relative electron paramagnetic spin resonance

(EPR) signal of isolated rat hearts (Chapter 5). This is in line with previous studies of animals exposed to PM (Gurgueira *et al.*, 2002; Rhoden *et al.*, 2005). The DEP-instilled rats also showed a greater loss of viable myocardium prior to the induction of ischaemia, as determined by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining (Chapter 5). Parallel experiments confirmed that the loss of viability observed by TTC was due to apoptosis (Appendix 4). The abrogation of the DEP-induced cardiac oxidative stress and cell death by pre-treatment with metoprolol, a $\beta_1$ adrenoceptor antagonist, supports the involvement of $\beta_1$-adrenoceptors in eliciting the observed response (Chapter 6). This agrees with previous studies that have shown that enhanced adrenoceptor signaling induces oxidative stress and cell death in cardiac myocytes (Communal *et al.*, 1998; Park *et al.*, 1999; Romeo *et al.*, 2000). In the heart $\beta_1$-adrenoceptors predominate and participate in efferent sympathetic cardiac regulation (Grimm *et al.*, 2010; Wallukat, 2002). Sympathetic nerve activity was not measured directly and needs to be confirmed in subsequent experiments (for example by microneurography (Nakamura *et al.*, 2003)). No significant differences were found for plasma catecholamine levels measured 6 hours after the instillation of DEP compared with saline control rats (Chapter 4). The accurate measurement of catecholamines is, however, notoriously difficult and all animals were likely stressed from the handling and moving of cages (Kvetnansky *et al.*, 1978). Direct effects of blood-borne particles on the heart and/or from indirect consequences of DEP-induced pulmonary inflammation cannot be completely ruled out. The later explanation seems unlikely since there was no evidence that DEP induced plasma cytokines or granulocyte activation or priming (Chapters 4 & 5). Similarly, direct effects of DEP on the heart are unlikely based on previous calculations of the amount of UFP that could access the bloodstream (<1%) (Kreyling *et al.*, 2002). Instead it seems a lot more likely based on the participation of $\beta_1$-adrenoceptors, coupled with the lack of effect of DEP exposure on systemic inflammation or on the oxygen carrying capacity of the blood (no change in red blood cell (RBC) count), that sympathetic activation is a major determinant of the cardiac oxidative stress and cell death. Furthermore, the abrogation of both DEP-induced cardiac oxidative stress and cell death by metoprolol pre-treatment provides support that the oxidative stress and reduction in cell viability are closely linked events. Indeed oxidative stress is well

recognized as a major contributing factor to the induction of apoptosis (Akki *et al.*, 2009).

Data from Rhoden and colleagues showed that short-term exposure to CAP increased oxidative stress in the rat heart, which could be prevented by pre-treatment with atenolol (a $\beta_1$ adrenoceptor antagonist) (Rhoden *et al.*, 2005). These results further indicate that cardiac oxidative stress may result from an imbalance in cardiac autonomic activity in response to deposition of PM in the lung. Reactive oxygen species (ROS) have been shown to be mediators in this pathway (Rhoden *et al.*, 2005). ROS levels in the systemic circulation were not measured in the current study. However, granulocytes, and especially neutrophils, are a major source of ROS. In the present study, although the number of white blood cells (WBC) increased 6 hours after DEP exposure, granulocytes were not activated (CD11b expression) (Chapter 5). Moreover, ROS are known to induce cytokines and vice versa (Stocker *et al.*, 2004; van Eeden *et al.*, 2002; Yoshida *et al.*, 1999). As mentioned above, there was no increase in circulating cytokine levels found in rats 6 hours after exposure to DEP. Collectively, these data suggest that ROS in the systemic circulation does not contribute to the occurrence of cardiac oxidative stress following DEP exposure. It would be useful in future studies to measure oxidative stress indicators, such as glutathione (GSH) in blood. An alternative approach would be to evaluate whether pre-treatment of rats with an anti-oxidant, such as N-acetyl-cysteine (NAC), could prevent the cardiac oxidative stress.

It should be emphasised that it remains unclear whether the increased cardiac oxidative stress is due to increased ROS production and/or a reduced level of activity of anti-oxidants. The lack of neutrophil infiltration on haematoxylin and eosin (H & E)-stained paraffin sections from hearts of rats instilled with DEP 6 hours previously did, however, rule out neutrophils as major sources of ROS. Based on the results discussed above, it would be interesting to evaluate the effects of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibition, a major source of ROS (Jordan *et al.*, 1999), on the DEP-induced cardiac oxidative stress. NADPH oxidase is widely distributed within the heart (Cave *et al.*, 2005) and increased NADPH

oxidase-derived ROS production has been reported upon alpha 1 ($\alpha_1$) adrenoceptor activation (Xiao *et al.*, 2002). Furthermore, the involvement of NADPH oxidase in PM-induced oxidative stress has been reported both *in vivo* (Kampfrath *et al.*, 2011) and *in vitro* (Mo *et al.*, 2009). Using pharmacological inhibitors of NADPH oxidase would also help to verify whether an oxidative mechanism is involved in the myocardial apoptosis.

In this study, multiple lines of evidence pointed towards impaired modulation of autonomic regulation, partly as a result of activation of vagal afferent C-fibers innervating the lung rather than as a consequence of DEP-induced pulmonary and systemic inflammation. Blocking the $\beta_1$-adrenoceptors did not completely abrogate the DEP-induced pulmonary inflammation (Chapter 6). As already mentioned above, exposure to DEP did not enhance the adhesive properties of neutrophils or increase levels of circulating pro-inflammatory cytokines. Finally, the $\beta_1$-adrenocpetor-mediated myocardial cell death following DEP exposure was also prevented by pre-treatment with the transient receptor potential vanilloid 1 (TRPV1) inhibitor AMG 9810 (Chapter 6 and Appendix 5). Activation of sensory receptors in the airways has been reported to modify sympathetic and parasympathetic output via feedback to the central nervous system (CNS) (Widdicombe *et al.*, 2001). Indeed, pharmacological studies support a role for TRP receptor activation *in vivo* to PM-induced cardiac oxidative stress and electrophysiological changes (Ghelfi *et al.*, 2008). Rather unexpectedly, the TRPV1 inhibitor AMG 9810 did not prevent the DEP-induced cardiac oxidative stress (Chapter 6). The reasons for this are not obvious but may simply be a reflection of insufficient statistical power to detect a meaningful difference. Here it is worth mentioning that it is not clear if AMG 9810 blocks all pools of TRPV1. Indeed, distinct pools of TRPV1 within cells have been linked to different downstream effects (Reilly *et al.*, 2005). However, replication studies with a larger sample size are needed before any firm conclusions can be drawn.

It is also noteworthy, that pharmacological blockade of TRPV1 had partial, but significant, effects on DEP-induced lung inflammation (Chapter 6). This may be explained by attenuation of the local axon reflex-mediated neurogenic inflammation

in the lung (Holzer, 1988) by blocking the activation of TRPV1. This strongly suggests that the inflammation elicited by DEP is due, in part, to neurogenic inflammation. Conflicting results have suggested that the C-fibre-mediated axon reflex plays no major role or plays the primary role in PM-induced inflammation (Oortgiesen *et al.*, 2000; Veronesi *et al.*, 1999b; Witten *et al.*, 2005; Wong *et al.*, 2003). These findings also reveal insight into whether TRPV1 detects DEP directly or indirectly (via inflammatory mediators). The inflammatory response in association with PM exposure has been reported to activate TRP receptors (Bessac *et al.*, 2008). However, the abrogation of DEP-induced injury as well as sensitisation to reperfusion injury suggests that DEP may be able to bind and activate TRPV1 directly. This conclusion is supported by a recent study showing that DEP directly activates TRPV1 (Deering-Rice *et al.*, 2011). Direct activation of TRPA1 by DEP was also confirmed (Deering-Rice *et al.*, 2011) highlighting the need to address the potential contribution of other channels in mediating the observed effects on cardiac injury.

## 7.4 DEP exposure increases susceptibility to myocardial I/R injury both *ex vivo* and *in vivo*

A single exposure of the lung to DEP 6 hours prior to I/R increased myocardial sensitivity to I/R injuries, most notably characterised by increased susceptibility to ischaemia-induced arrhythmias and arrhythmic death as well as an increase in the infarct size (Chapter 5). This study is clinically relevant, particularly in light of the recent clinical study reporting an increased risk for myocardial infarction (MI)-associated hospitalisation in the first 6 hours after exposure to atmospheric pollution (Bhaskaran *et al.*, 2011). The increased susceptibility to arrhythmias may be associated with increased cardiac metabolic demand during ischaemia (increased rate pressure product (RPP); Chapter 4). Alternatively, the oxidative stress induced by DEP before I/R may have decreased the capacity of the heart to deal with the arrhythmogenic effect of a subsequent ischaemia. It has been reported that pre-treatment with the anti-oxidant NAC was capable of blocking arrhythmias *in vivo* following exposure to DEP (Kim *et al.*, 2012). Furthermore, these investigators showed that oxidative stress caused by exposure to DEP induces arrhythmias by

calcium ($Ca^{2+}$)/calmodulin kinase II (CaMKII) activation (Kim *et al.*, 2012). $\beta_1$ adrenoceptor-evoked increases in intracellular $Ca^{2+}$ has also been associated with arrhythmias via activation of CaMKII (Anderson, 2007; Grimm *et al.*, 2010). Moreover, sympathetic drive is a key regulator of cardiac rhythm and the SNS has been implicated in mediating alterations in heart rate variability (HRV) (Ghelfi *et al.*, 2008; Hazari *et al.*, 2011). Increased aconitine-induced arrhythmia sensitivity after whole diesel exhaust (wDE) exposure was fully prevented by guanethidine (a sympathetic blocking drug) (Hazari *et al.*, 2009). Future studies using β adrenoceptor blockers are necessary to establish whether SNS activation contributes to the increased susceptibility to ischaemia-induced arrhythmias in DEP-instilled rats. In addition to sympathetic activation, Hazari and colleagues also demonstrated a role for transient receptor potential ankyrin 1 (TRPA1) in mediating the heightened arrhythmia sensitivity following inhalation of wDE (Hazari *et al.*, 2011). In the present study, pre-treatment with a TRPV1 antagonist completely abrogated DEP-induced loss of cell viability, as well as sensitisation to injury *ex vivo* (69% inhibition in infarct size). Treatment of rats with TRPV1 inhibitors before DEP exposure is needed to ascertain the role of TRPV1 in the ischaemic arrhythmias. It will also be necessary to investigate the effects of TRPV1 blockade on the ANS function in DEP-instilled rats. Furthermore, assessing arrhythmias in *ex vivo* perfused hearts of DEP-instilled rats will specifically determine whether *in vivo* mechanisms affect the incidence of arrhythmias during the ischaemia and reperfusion.

It has been reported that granulocyte (especially neutrophil) infiltration plays an important role in the development of arrhythmia and cell death due to I/R (Jordan *et al.*, 1999). Based on the flow cytometry data (Chapter 5), DEP exposure did not alter the activation or priming responses of granulocytes. Although not assessed in this study, neutrophil recruitment to the myocardium during I/R may underlie the increased arrhythmogenic susceptibility, as has been shown by others (Yokota *et al.*, 2004; Yokota *et al.*, 2008a). I/R conducted on isolated hearts from rats exposed to DEP did, however, show that neutrophil infiltration during I/R was not essential for mediating the increased infarct size. The heightened sensitivity to I/R-induced injury *in vivo* and *ex vivo* also demonstrated that hearts are primed *in vivo* following

exposure to DEP. Additionally, studies using pharmacological inhibitors of $\beta_1$ adrenoceptors and TRPV1 confirmed that activation of pulmonary TRPV1 and systemic $\beta_1$ adrenoceptors are central to the heightened sensitivity to I/R-triggered cell death *ex vivo*. As mentioned above both these receptors were shown to be implicated in the heart injuries (oxidative stress and cell death) induced by DEP exposure alone. Collectively, these data confirm that this priming effect upon exposure to DEP was associated with increased oxidant stress generation from the heart, accompanied by loss of cardiomyocyte viability. Furthermore, activation of pulmonary TRPV1 and systemic $\beta_1$ adrenoceptors appear to be necessary for priming of the heart for subsequent injury. As mentioned above DEP-induced lung inflammation was not completely blocked by the TRPV1 antagonist revealing that the mechanism by which hearts are primed is at least partially independent of lung inflammation. This was further confirmed by a complete reversal of the heightened sensitivity to myocardial reperfusion injury, but not lung inflammation, in DEP-exposed animals by blockade of $\beta_1$ adrenoceptors. Repeating the drug intervention studies using an *in vivo* model of I/R is needed to confirm or refute whether $\beta_1$ adrenoceptor blockade and/or TRPV1 blockade prevents the increased I/R-triggered cell death in the DEP-instilled rats.

## 7.5 General considerations

All studies used DEP. The physiochemical properties of DEP vary depending on the fuel used and the engine type and running conditions. The work described in this thesis used widely available 'standard' DEP from the National Institute of Standards and Technology (NIST), a frequently used source of DEP that acts as a benchmark for comparisons of the biological effects of DEP between studies. It is important to acknowledge that although the dose used was lower than that used in other studies (Yokota *et al.*, 2004; Yokota *et al.*, 2008a) it was substantially higher than that expected from real-world exposures. The total dose delivered to the alveolar surface was approximately 30-fold higher than the comparable alveolar deposition from a 24 hour inhalation of 100 $\mu g/m^3$ in man (multiple path particle dosimetry (MPPD) model, adjusted rat lung surface area) (Cassee *et al.*, 2002). Nevertheless, this does not, for instance, take into consideration peak PM levels which occur regularly,

particularly in large cities, or the increased PM deposition during exercise (e.g., brisk walking, cycling or jogging) (Daigle *et al.*, 2003). The research described in this thesis was conducted in healthy rats. It is unclear whether biological responses will be evident at lower doses among specific sub-groups of the population, such as those with pre-existing cardiovascular disease. Indeed it has been reported that certain sub-groups of the population are more susceptible to health effects from exposure to PM (Daigle *et al.*, 2003; Miller *et al.*, 2007).

Lastly, intra-tracheal instillation was used to deliver particulate to the lungs of rats. This approach has been successfully used previously to investigate the health effects of DEP on the cardiovascular system (Madden *et al.*, 2000; Murphy *et al.*, 1998; Yan *et al.*, 2008; Yokota *et al.*, 2004; Yokota *et al.*, 2008a; Yokota *et al.*, 2005; Yokota *et al.*, 2008b). Although intra-tracheal instillation is less physiological than inhalation, it has been widely accepted as a reliable technique to administer particles directly to the respiratory tract in a quantitative and reproducible manner (Driscoll *et al.*, 2000; Miyabara *et al.*, 1998). In comparison to inhalation exposure, instillation results in better dispersion of particles throughout the lobes of rodent lungs and across the alveolar surface (Driscoll *et al.*, 2000; Miyabara *et al.*, 1998). The nasal passages of rodents are more complex than humans and serve as a filter for the inhaled particles. This filtering of particulates reduces the quantity of particles able to reach the alveoli and limits dose reproducibility (Driscoll *et al.*, 2000; Miyabara *et al.*, 1998). Additionally, PM has been reported to induce similar increases in bronchoalveolar lavage (BAL) fluid cell counts for both methods of exposure (Driscoll *et al.*, 2000; Henderson *et al.*, 1995; Miyabara *et al.*, 1998).

## 7.6 Main conclusions

Airway inflammation, characterised by increased protein permeability, neutrophil infiltration and increased production of inflammatory cytokines, was detectable at 6 hours after exposure to DEP. At this time-point, hearts from DEP exposed rats were more sensitive to I/R injury *in vivo*, indicated by increased susceptibility to ischaemia-induced arrhythmias and arrhythmic death as well as an increase in infarct size. No endothelial dysfunction or systemic inflammation was detected 6 hours

after exposure and, thus, these processes are unlikely to be causally related to the increased ischaemic vulnerability. Isolation of hearts 6 hours following exposure to DEP demonstrated that exposure primed the hearts for subsequent injury and that enhancement of inflammatory cell recruitment was not required. Priming was associated with increased oxidant stress generation from the heart, accompanied by loss of cardiomyocyte viability. Co-administration *in vivo* of a $\beta_1$ adrenoceptor antagonist completely abrogated the DEP-induced cardiac injury as well sensitisation to injury *ex vivo*, suggesting that activation of the SNS was key in the priming mechanism. The increased blood pressure 6 hours after DEP exposure was also most probably due to increased sympathetic activation. Sensory transduction involving TRPV1 channels in the lungs feeds back to the CNS inducing changes in autonomic outflow to the heart (Widdicombe *et al.*, 2001). A role for this pathway was strongly supported by the observation that myocardial priming by DEP could be prevented when pulmonary TRPV1 receptors were blocked using a selective antagonist. Together these data implicate activation of a sensory feedback loop by DEP in the lung leading to increased sympathetic outflow and priming of the heart for increased ischaemic injury (Figure 7.1). This mechanism may also provide a plausible explanation for recent reports associating short-term increases in exposure to PM (within 1-6 hours) with 'short-term displacement' of myocardial events rather than increasing overall risk (Bhaskaran *et al.*, 2011).

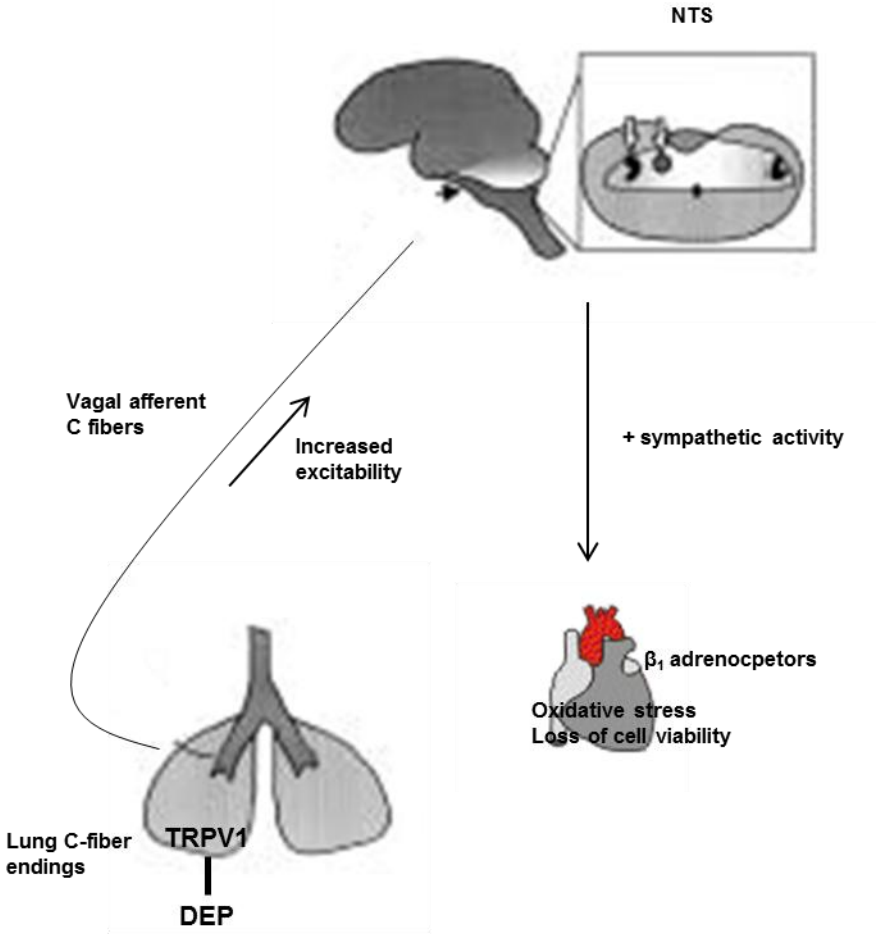


**Figure 7.1 Schematic of proposed mechanism for the transmission of effects of diesel exhaust particulate (DEP) in the lung to the heart.**

Airway activation of transient receptor potential vanilloid 1 (TRPV1) sends an afferent signal to the nucleus tractus solitaries (NTS) in the brain stem, and in turn, increases efferent sympathetic outflow to the heart. The sympathetic nervous system (SNS) acts via beta 1 ($\beta_1$) adrenoceptors to induce oxidative stress and cell death that prime the heart for subsequent injury. Figure adapted from Bonham *et al*., 2001.

# Chapter 8

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# Appendix 1: Supplemental materials and methods

## *Ex vivo* vascular function using myography

The thoracic aorta, a region of un-injured femoral artery and 3rd order mesenteric resistance arteries were isolated from animals 6 or 24 hours after instillation of diesel exhaust particulate (DEP, 0.5 mg in 0.5 ml saline) or vehicle (saline 0.5 ml), and cleaned of connective tissue. Segments of aorta (~5 mm length), femoral and mesenteric arteries (both 1-2 mm length) were mounted in a multi-myograph system (610M; Danish Myo Technology, Aarhus, Denmark) (Miller *et al.*, 2009), using 40 μm diameter wire for smaller vessels. Vessels were submerged in Krebs buffer (composition in mM: 118.4 NaCl, 25 $NaHCO_3$, 11 glucose, 4.7 KCl, 1.2 $MgSO_4$, 1.2 $KH_2PO_4$, 0.027 ethylenediaminetetraacetic acid, 2.5 $CaCl_2$) bubbled with 5% $CO_2$/95% $O_2$ at 37°C before a baseline tension of 14.7 mN (aorta), 8 mN (femoral) or 4 mN (mesenteric) was gradually applied over 10 minutes and vessels were allowed to equilibrate for a further 30 minutes. Preliminary experiments showed that these pre-tension levels produced optimal contraction and dilatation responses. Data from force transducers were processed by a MacLab/4e analogue-digital converter displayed through Chart™ software (AD Instruments, Sussex, UK).

Vessel viability was confirmed by a contractile response on addition of 80 mM KCl, repeated 3 times (aorta) or serial addition of high $K^+$ Krebs (Krebs with substitution of 4.7 mM NaCl and 118.4 mM KCl) together with 10 μM noradrenaline (femoral and mesenteric arteries). Concentration-response curves to phenylephrine (PE; 1 nM – 10 μM) were obtained and a concentration that produced 80% maximum contraction ($EC_{80}$; 0.1-1 μM) was chosen for each individual arterial segment. Following sub-maximal contraction with the appropriate concentration of PE, cumulative concentration-response curves were obtained for acetylcholine (ACh; 1 nM - 10 μM), sodium nitroprusside (SNP; 0.1 nM - 3 μM) and a nitric oxide-

independent vasodilator (isoprenaline; 0.1 nM - 10 μM). A 30 minute washout was allowed before application of subsequent drugs.

Results are expressed as percentage of the pre-contraction to $EC_{80}$ PE, where positive values represent vasodilatation and 100% vasodilatation represents a complete abolition of PE-induced tone. Statistical comparisons were performed by two-way ANOVA using the Bonferroni post-hoc test.

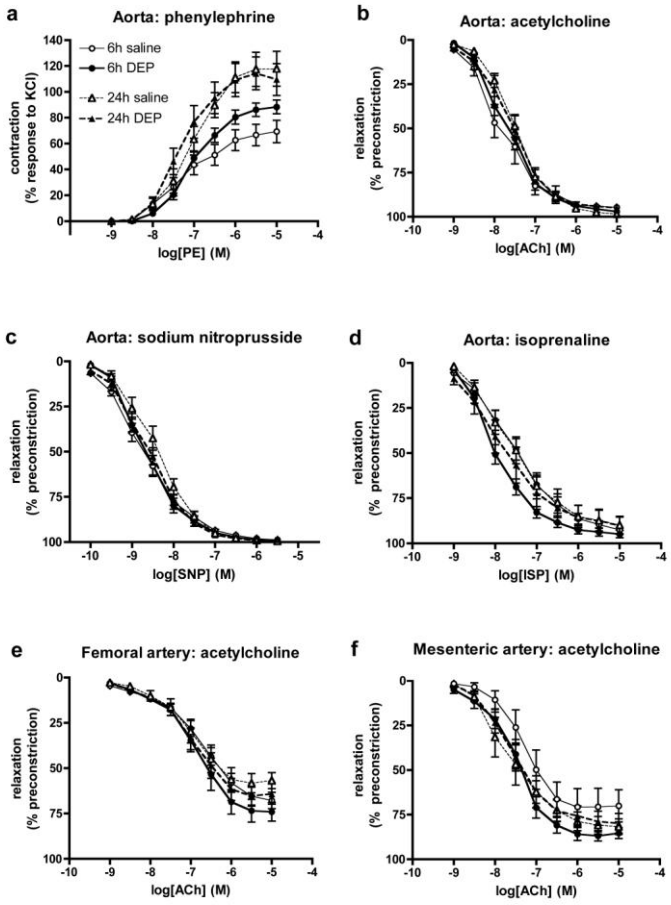
## Immunohistochemistry using TACS® 2 TdT-Blue Label in situ Apoptosis Detection Kit

Apoptosis in heart tissue samples were evaluated using a **T**erminal Deoxynucleotidyl Transferases d**U**TP **N**ick **E**nd **L**abeling (TUNEL) assay according to the manufacturer's instructions (TREVIGEN®, Gaithersburg, USA). Briefly, heart tissue was collected from rats after no instillation and 6 hours after intra-tracheal instillation of DEP (0.5 mg in 0.5 ml saline) or vehicle (saline 0.5 ml) alone or from rats that had received metoprolol (10 mg/kg, intraperotineal) or AMG 9810 (30 mg/kg, intra-tracheal) at the time of instillation *in vivo*. The hearts were fixed for 24 hours in 10% neutral buffered formalin prior to further processing and paraffin wax embedding as described in Chapter 2.8. Paraffin wax-embedded hearts were sectioned at 5 μm thickness, then deparaffinized and rehydrated. Please refer to Chapter 2.9.2 for more details. Sections were then incubated for 10 minutes in phosphate buffer saline (PBS). Slides were placed in a magnetic immunology staining tray and covered with 50 μl Proteinase K solution for 30 minutes at 37°C. After washing in deionised water (2 changes of 2 minutes), sections were immersed in Quenching solution (5 minutes) followed by washes in PBS (3 changes of 1 minute). Sections were then placed in terminal deoxynucleotidyl transferase (TdT) labeling buffer for 5 minutes and covered with 50 μl of labeling reaction mix to allow TdT-mediated dUTP nick end labeling. After incubation for 1 hour at 37°C, the reaction was stopped by incubation in TdT stop buffer for 5 minutes. Following washes in PBS (2 changes of 5 minutes), modified nucleotides were visualized under a light microscope using TACS- Blue Label™ (approximately 3 minutes for colour

development). After washing in deionised water (2 changes of 2 minutes), sections were counterstained with Nuclear Fast Red (2 minutes), followed by a wash in deionised water and dehydration through a graded series of alcohol (74% ethanol for 1 minute twice, 100% ethanol for 1 minute). Finally, a cover slip was mounted using DPX mounting media. A negative control was included by omitting TdT from the reaction mixture.

TUNEL-positive cells were viewed with an Image Pro6.2, Stereologer Analyser 6 MediaCybernetics (Buckinghamshire, UK). For quantification, sections were tiled at x100 magnification (using a 100x objective). The entire left ventricle (LV) was identified as an area of interest using the Image Pro6.2 computer programme after image acquisition. 30 randomly selected areas of 2 $mm^2$ per section were examined. Results are expressed as the mean number of TUNEL-positive cells per 2 $mm^2$ ± SEM. One tissue section per rat heart was counted. Data was analysed using one-way ANOVA combined with Bonferroni's post hoc test for multiple comparisons.

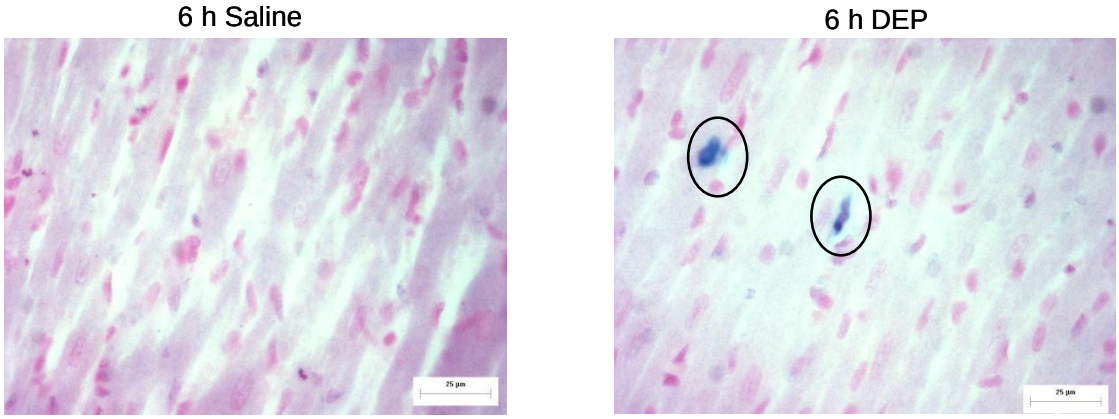
# Appendix 2: *Ex vivo* vascular function




**Instillation of diesel exhaust particulate (DEP) has no effect on vascular responses *ex vivo.***

**(a)** Contraction to phenylephrine (PE), and relaxation to **(b)** acetylcholine (ACh), **(c)** sodium nitroprusside (SNP) and **(d)** isoprenaline (ISP) in the thoracic aorta. Responses to ACh in **(e)** femoral artery and **(f)** 3$^{rd}$ order mesenteric arteries. Data from saline-instilled animals (open symbols) and DEP-instilled animals (closed symbols) sacrificed at 6 hours (circles, solid line) or 24 hours (triangles, dashed line) after instillation. Results are expressed as mean ± SEM (n=6-10). No significant differences were found between saline- or DEP-instilled animals at either time points for all vessels (two-way ANOVA).

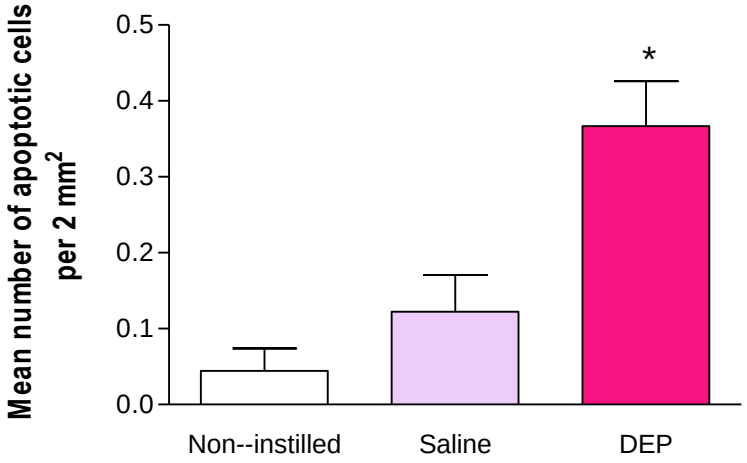
# Appendix 3: Left ventricle TUNEL staining of DEP- and saline-instilled rats: microscopic images




**Representative images of TUNEL-stained left ventricular sections showing positive apoptotic cells collected 6 hours after instillation with diesel exhaust particulate (DEP).**

Left ventricle (LV) myocyte TUNEL staining 6 hours after instillation of saline or DEP (0.5 mg). (Scale bar = 25 μm).

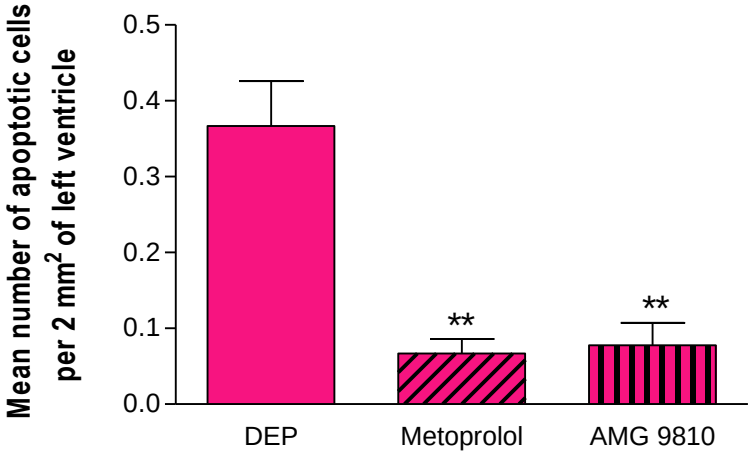
# Appendix 4: Left ventricle TUNEL staining from non-instilled, saline and DEP-instilled rats




**Diesel exhaust particulate (DEP) instillation increased cardiac myocyte apoptosis.**

TUNEL-positive cell numbers per 2 $mm^2$ in the left ventricle (LV) of hearts from non-instilled, saline-instilled and DEP-instilled (0.5 mg) rats 6 hours after instillation. Columns represent mean ± SEM (n=3-4) *P<0.05, compared to saline; one-way ANOVA with Bonferroni post-hoc test.

# Appendix 5: Effects of drug intervention on apoptosis of hearts from DEP-instilled rats




**The $\beta_1$ adrenoceptor antagonist metoprolol and the TRPV1 receptor antagonist AMG 9810 prevented diesel exhaust particulate (DEP)-induced cardiac myocyte apoptosis.**

TUNEL-positive cell numbers per 2 $mm^2$ in the left ventricle (LV) of hearts isolated 6 hours after instillation with DEP (0.5 mg) alone (open columns); in hearts from rats that received metoprolol at the time of instillation *in vivo* (10 mg/kg, intraperotineal, hatched columns); or in hearts from rats that received AMG 9810 at the time of instillation *in vivo* (30 mg/kg, intra-tracheal, vertical columns). Columns represent mean ± SEM (n=3-4) **P<0.01, compared to saline; one-way ANOVA with Bonferroni post-hoc test.